Behavioural effects of grafts of two areas of the basal forebrain and their combination after fimbria-fornix lesion.

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A thesis submitted in partial fulfilment of the requirements of a Master of Science degree in Psychology.

1995.

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Acknowledgements.

The author would to thank Dr. John Dalrymple-Alford for his time, humour, enthusiasm and availability. Thank you also to Mrs. Patricia Meatchem, the life blood of level 6, for technical assistance. Thank you to Robin Phillips for developing my photographs so wonderfully, and thank you to Howard Patterson and Glen Lewis for constructing and maintaining apparatus.

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Table and Figures.

Abstract.

Adult rats received bilateral fimbria-fornix/cingulate cortex lesions followed by intrahippocampal (12mm CRL) suspensions of either Lateral Basal Forebrain (LBF), Ventromedial Basal Forebrain (VBF) or a mixture of both tissues (group MBF), equated for cell density. Relative to lesion-only controls, there was no amelioration in any graft group of the lesion-induced working memory impairment in a T-maze task. Postoperative reacquisition of reference memory performance, however, was significantly impaired only in group LBF by comparison with intact controls; the other three lesion groups showed an intermediate level of performance. By comparison with intact controls, all four lesion groups were also impaired on DRL-20 and subsequent DRL-10 tests. Unlike the DRL-20 test, lesioned rats showed improvement across sessions on the DRL-10 test; relative to intact controls, the lesion-only group's final performance on DRL-10 was not significantly impaired whereas as all three grafted groups remained significantly impaired throughout training. Lesion-induced spontaneous hyperactivity was not affected by graft status. Surviving implants were found in all grafted rats, with moderate to excellent cholinergic reinnervation present in groups VBF and MBF. However, weak or virtually absent cholinergic reinnervation was present in group LBF, with relatively poor AChE-positivity outside the region of the graft tissue. This study provides further evidence that basal forebrain grafts, irrespective of any associated cholinergic innervation of the hippocampus, may not improve lesion-induced behavioural deficits.

Introduction.

A number of factors make neural transplantation an attractive proposal: neurones lost from the Central Nervous System (CNS), for what ever reason, are not replaced, making spontaneous recovery from progressive neuro-degenerative disorders such as Alzheimer's Disease impossible; the CNS, with its limited immune responsiveness, is a privileged site for transplantation of "foreign" material; foetal neural cells are remarkably robust, many cells being able to withstand oxygen deprivation for hours apparently without ill effect; and studies using transplants of such foetal neural material demonstrate that it can survive, grow, form axonal connections, and even ameliorate functional and neurochemical impairments (Bjorklund, 1991; Cassel et al, 1992, Dunnett et al, 1982a). Together, these factors make the potential therapeutic benefits of neural transplantation enormous.

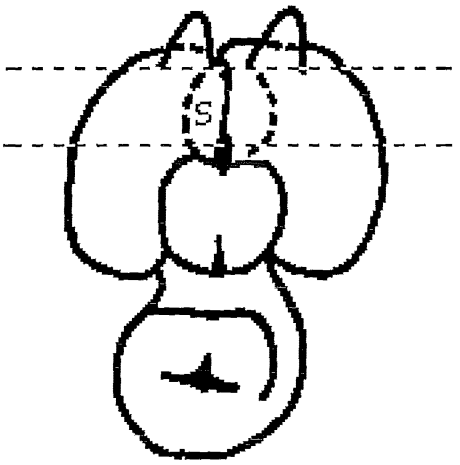
The present study considered the potential of neural grafts of two areas of the developing basal forebrain to facilitate recovery of function in an animal lesion model of an important component of the brain damage that occurs in Alzheimer's disease.

Septo-hippocampal pathway lesions.

The dementia of Alzheimer's Disease may be attributed to the decline in cortical cholinergic activity (Bartus et al, 1982; Coyle et al, 1983) and to the

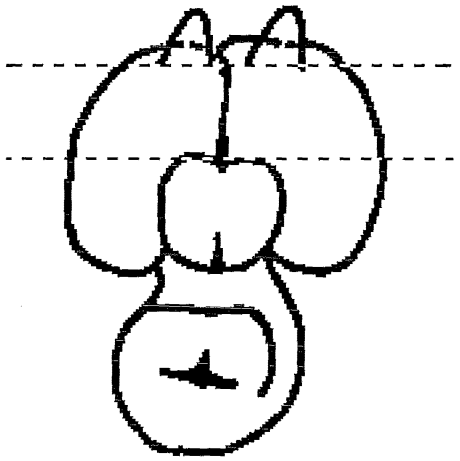
loss of subcortical neurones of the nucleus basalis of Meynert (Candy et al, 1983). This central role of the cholinergic system in Alzheimer dementia has lead to the development of the "cholinergic hypothesis of memory dysfunction", and despite problems the hypothesis has much evidential support. Functional deficits in cases of dementia have been shown to correlate with low postmortem levels of choline acetyltransferase (ChAT) (Bartus et al, 1982). Decline in memory function in normal subjects given scopolamine, a cholinergic antagonist, also suggests a cholinergically mediated memory system (Drachman and Sahakian, 1980). Alzheimer type dementia patients reliably show exaggerated bi-directional effects of cholinergic drugs further supporting the cholinergic hypothesis (Sahakian et al, 1989).

The hippocampus is functionally associated with cognitive abilities such as memory (Olton, 1983), and appropriate lesions of the septo-hippocampal system in animals, namely lesions of the cholinergic projections, cause impairments in learning and memory very similar to those found in Senile Dementia of the Alzheimer Type (SDAT). Such lesioned animals also display exaggerated effects of cholinergic drugs in a manner in many ways analogous to humans with SDAT (Ridley et al, 1986; Tilson et al, 1988; Nilsson and Gage, 1993). The fimbria-fornix is the primary cholinergic afferent pathway of the hippocampus, and transection of the fimbria-fornix creates an SDAT condition in animals which effectively models many aspects of the behavioural and neuropathological deficits associated with SDAT in humans (Dunnett, 1991).

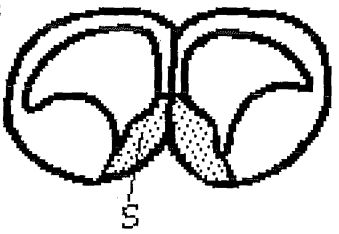


(a) Ventral view of a 15mm foetal rat brain showing extent of coronal section used in (c), and (d).

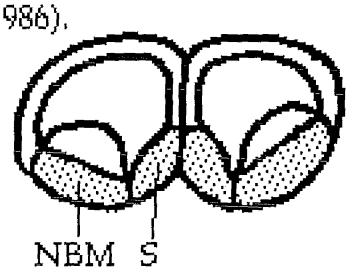
(b) Below, shows the extent of the coronal section used in (e), (f) and (g).



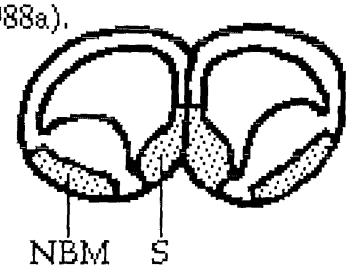
(c) Standard graft of basal forebrain.



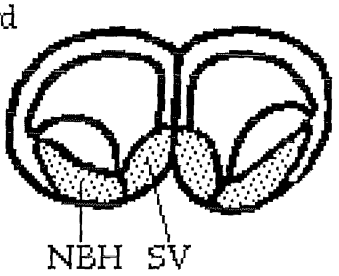
(d) Dunnett et al (1986).



(e) Nilsson et al (1988a).



(f) Dalrymple-Alford (in prep.).



(g) Webster & Dalrymple-Alford, (12/13mm CRL).

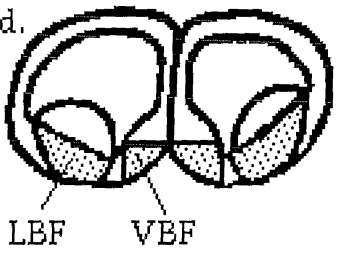


Figure 1. Differential dissection schemes of the coronal section. Abbreviations: S - septal nucleus, NBM - area containing nucleus basalis magnocellularis cells, NBH - nucleus basalis including the horizontal part of the diagonal band of Broca, SV - septal nucleus including the vertical part of the diagonal band of Broca, LBF - lateral basal forebrain, VBF - ventromedial basal forebrain.

Neural transplants

Neural grafts of cholinergically rich foetal neural tissue have been shown to reinnervate the host hippocampal formation in rats with fimbria-fornix lesions (Bjorklund and Stenevi, 1977; Dunnett, 1991). Cholinergic transplants are known to ameliorate a variety of functional deficits associated with lesions and the normal aging process. The tissue known to yield the best restoration of cholinergic neurochemical markers is the developing cholinergically rich ventro-medial basal forebrain - this being the very tissue that includes cells which grow to form the cholinergic system in the adult (Dunnett, 1991).

The "standard" septal transplantation technique involves dissecting out the ventro-medial area of the rostral foetal forebrain, approximately the bilateral area marked "S" in figure 1a. This dissection is done either directly from the intact foetal brain, or via a coronal slab of tissue taken at an angle approximately perpendicular to the ventral surface, an area as indicated by the hatched lines in figure 1a. Figure 1c shows the septal area used for transplantation in the standard method as described by Dunnett et al (1986). The step involving a coronal section is used in an attempt to isolate more accurately the piece of foetal tissue containing the desired cell populations. However, most work dissects out the basal forebrain area without making use of a coronal section, often making it unclear precisely which tissue area is actually used. Foetal tissue, once attained, can either be transplanted into the adult host as a solid mass, or as gives better integration results, made into a suspension and stereotaxically injected into host target sites, usually ectopically into the hippocampus.

There is much evidence to support the effectiveness of both septal transplantation as a means of neurochemical and behavioural restoration

and fimbria-fornix transection as a model of the degenerative processes of dementia. For example, septal grafts have been shown to ameliorate spatial navigation deficits shown by aged rats tested in the Morris (1981) water maze (Dunnett, 1991). Dunnett et al (1982b) found 11 out of 14 young adult rats with septal grafts achieved criterion performance on a T-maze task, while lesion-only controls did not; this recovery was also correlated with acetylcholinesterase-(AChE)-positive reinnervation of the hippocampus. Dunnett et al (1989) found recovery of differential reinforcement of low rates (DRL) responding after fimbria-fornix lesion and standard septal grafts. Nilsson et al (1987) demonstrated restoration of normal Morris water maze behaviour in 60-80% of rats after fimbria-fornix lesions and standard septal grafts. Nilsson et al also reported that this behavioural recovery could be abolished by the administration of the cholinergic antagonist atropine, lending further support to the idea that such behavioural deficits and recovery are cholinergically mediated.

In many studies, however, the precise factors affecting either neurochemical or behavioural recovery remain unclear. There are conflicting reports regarding the behavioural effects of intrahippocampal basal forebrain grafts, and the relationship between cholinergic reinnervation and behavioural recovery. Such conflicting findings make generalisations in the field difficult.

While many studies have found graft-induced behavioural improvements which have been correlated with cholinergic reinnervation (Dunnett et al, 1982b), transplantation of neuroblastoma cells to the hippocampus may improve T-maze task performance despite a lack of cholinergic reinnervation (Kordower et al, 1987). Even cholinergically rich basal forebrain grafts may improve some cognitive deficits despite poor AChE-positive fibre reinnervation of the

hippocampus (Emerich et al, 1992). Others have found restoration of cholinergic markers but only a modest behavioural recovery dependent on a particular lesion-graft delay (Dunnett et al, 1989), the age of the donor tissue (Cassel et al, 1991; Dunnett et al, 1989), or the particular behavioural measure used to assess recovery (Cassel et al, 1991; Dunnett et al 1989; Gasbarri et al, 1993).

In addition, some studies have found deleterious effects of basal forebrain grafts. Dunnett et al (1982b) commented that some rats experienced additional impairments relative to non-graft controls on the Morris (1981) water maze task after septal transplants. Dalrymple-Alford et al (1988) reported deficits in serial alternation and radial maze performance which were more severe than those shown by lesion-only counterparts in rats with partial fimbria-fornix lesions and septal transplants.

Despite these conflicting results a number of generalisations have been made about the findings of basal forebrain neural transplants which seem reliable. First, younger tissue of the age ~13 mm Crown-Rump Length (CRL) seems more likely to give significant behavioural recovery from lesion impairment (Cassel et al, 1991; Dunnett et al, 1989). The precise reasons for this are unknown, but it is presumed to be something to do with the differential robustness of septal tissue as it develops, or vital ameliorative cell populations within that tissue. Second, a lesion-transplant delay of ~10 days seems to give optimal graft acceptance and ameliorative effect (Dunnett et al, 1989). A particular class of neurotrophic factors, wound-derived neurotrophic factors, are thought to be released in response to injury in the mammalian CNS, and while the level of these factors is low immediately after injury, they peak after ~10 days before declining again (Manthorpe et al, 1983; Nieto-Sampedro; 1982).

Transplantation during this peak period increases the chances of graft survival.

Differential dissection of transplant tissue.

Some interesting reinnervation results have been found by varying the standard septal dissection technique. Dunnett et al (1986) found that different areas of tissue from the ventral forebrain, namely the region containing the Nucleus Basalis Magnocellularis (NBM) cells, and the septal region (see figure 1d), gave different reinnervation results. Septal grafts produced better AChE-positive fibre outgrowth (a sign of graft integration) in a fimbria-fornix lesioned hippocampus (a fibre outgrowth volume of 20 mm^3) than in a nucleus basalis lesioned cortex (1 mm^3). While NBM grafts showed better AChE-positive fibre outgrowth in the denervated cortex (7 mm^3) than in the denervated hippocampus (4 mm^3). This was a highly significant location by tissue type interaction effect ($p < 0.001$). Since the NBM area normally projects to the cortex, while the septal area normally projects to the hippocampus, this difference in reinnervation seems to be one of cellular association or "appropriateness". Cells grow and integrate with surrounding tissue more effectively when implanted into a host area that those cells would normally innervate *in situ*.

Nilsson et al (1988a) with a slightly different differential dissection technique found quite different results to those of Dunnett et al (1986). Nilsson et al found that NBM grafts gave relatively good reinnervation in a denervated hippocampus (to ~50% normal levels), while septal grafts gave reinnervation comparable to normal levels. However, as can be seen in figures 1e and 1d (the author's interpretation of Nilsson et al's and

Dunnett et al's figures), what Nilsson et al call NBM tissue and what Dunnett et al call NBM tissue is not exactly the same area. In addition Nilsson's NBM tissue area appears made up almost entirely of cortex, which is known to be cholinergically poor (Schwaber et al, 1987), and so would be expected not to reinnervate even moderately. Why Nilsson et al then found reinnervation with NBM tissue, while Dunnett et al found relatively little is a puzzle. A possible explanation of this apparently contradictory finding maybe that Nilsson et al's actual NBM tissue areas included a cholinergically rich cell population, perhaps by including a more dorso-medial portion of the coronal section than their documentation would suggest. Dunnett et al may have missed this cholinergically rich cell population by using a coronal section which was more rostral. Figure 1a shows the extent of the coronal section used in Dunnett et al: notice the rostral position of the posterior incision relative to the hypothalamus. While figure 1b shows the extent of the coronal section used in Nilsson et al: notice that the coronal section extends further in the caudal direction.

Figure 1f shows a differential dissection scheme similar to Dunnett et al (1986) used by Dalrymple-Alford (in prep.) which attempts to separate more precisely the cholinergically rich basal forebrain cell populations which project to the cortex from those that project to the hippocampus. Using this scheme the diagonal band of Broca is separated into its vertical and horizontal parts. The vertical part of the diagonal band and the septal nucleus, which collectively project to the hippocampus, are included in tissue area SV, while the diagonal part and the nucleus basalis, which collectively project to the cortex, are included in tissue area NBH. Using these tissue areas Dalrymple-Alford performed intrahippocampal transplants on fimbria-fornix lesioned rats but found only very modest

behavioural recovery on a T-maze procedure (Helper et al, 1985) in the graft group which received NBH tissue. There was no evidence of behavioural recovery in the graft group which received SV tissue. The finding of behavioural recovery with NBH tissue but not SV tissue is a surprising one since tissue area SV contains the septal nucleus which is known to reinnervate a denervated hippocampus very well (Dunnett et al, 1986; Nilsson et al, 1988a), and to ameliorate lesion induced behavioural impairments (Dunnett et al, 1982b; Nilsson et al 1987).

Present study design.

Since there are many variables which seem to influence the success of neural transplants it seems reasonable that many of the apparently contradictory findings are situations where the desired effect has been confounded by some other uncontrolled-for variable (Cassel et al, 1992). A systematic teasing out of causal variables would greatly contribute to the field. However, many studies that have looked at the effects of different types of basal forebrain transplants have assessed graft success only in terms of the recovery of histochemical markers, and not in terms of the recovery of behavioural/cognitive function which is a vital aspect of recovery when assessing a system as intrinsically associated with memory as the cholinergic system (Olton, 1983). Similarly, assessment in terms of behavioural/cognitive function is highly relevant to possible therapy models for Alzheimer's Disease. To the author's knowledge, bar work in preparation by Dalrymple-Alford, no study using grafts from a differentially dissected basal forebrain has assessed recovery in terms of behavioural performance.

Based on preceding dissection schemes the coronal section in the present study (shown in figure 1g) is dissected so as to attain an area which more exclusively contains NBM cells (area LBF), and an area which more exclusively contains septal nucleus cells (area VBF). Given the reinnervation discrepancies between Dunnett et al (1986) and Nilsson et al (1988a), and the behavioural recovery discrepancies between Dalrymple-Alford (in prep.) and others (Dunnett et al, 1982b; Nilsson et al 1987) dissection method, and documentation of dissection method are obviously vital if the reinnervation and behavioural recovery properties of different basal forebrain areas are to be sorted out. For these reasons the present study attempts to make dissection as well defined as possible by using "landmarks" of the coronal section as guides for incisions, and by leaving small separating pieces of tissue between the two areas used for transplantation in an effort to keep the two cell populations separate. In addition, tissue area LBF is dissected so as to contain much less cortex than Nilsson et al (1988a); this is desirable since cortex is cholinergically poor. In addition, tissue area VBF is dissected so as to contain nearly all of the diagonal band of Broca cells, so as to be laterally comparable to Dunnett et al (1986), and concentrates on the cholinergically rich ventral cell population by having the more dorsal section of the septal nucleus not included.

The present study proposed to transplant tissue areas LBF, MBF and their combination intrahippocampally, and to do so in a way that would optimise graft effect. The present design includes an optimal lesion-transplant delay, and donor tissue of an optimal age. Three small 1.5 µl graft deposits are proposed in an attempt to yield the greatest possible reinnervation over the dorsal hippocampus, while reducing the

possibility of hippocampal disruption by the grafting of large volumes of foetal material.

The lesion proposed is a radiofrequency lesion of the fimbria-fornix and overlying cingulate cortex, as this is a lesion extensively used in comparative work (Nilsson et al, 1988a). Radiofrequency lesions are preferred since they cause less damage to surrounding structures and yield a more uniform lesion across subjects than aspirative lesions. The overlying cingulate cortex is lesioned in addition to the fimbria-fornix since this also contains a modest cholinergic afferent pathway (Gage et al, 1983).

The extensive behavioural testing used in the present study makes possible comparisons between cholinergic reinnervation status and behavioural recovery levels. In addition, the particular behavioural measures chosen were an attempt to tap a wide range of behaviours both to assess any behavioural recovery over a wide range of activities and to avoid any problems of task dependence with using a single behavioural measure.

Method.

Subjects.

The Forty female Wistar rats of an outbred strain, were 100 days old at the start of preoperative training. These animals were bred in the department's animal facility, housed in groups of four per cage, and maintained under a reversed 12 hour light-dark cycle (lights on between 0700 - 1900 h) in a temperature controlled colony room with water *ad libitum*. After each testing session the rats were fed a restricted diet to maintain 85% of *ad libitum* body weight throughout testing.

After preoperative training in the T-maze, subjects were randomly assigned on a matched basis to five groups, four of which received a bilateral lesion of the fimbria-fornix and the overlying cingulate cortex. Eleven to fifteen days later, three lesion groups received one of three different intrahippocampal grafts of basal forebrain tissue. The five experimental groups were: VBF (fimbria-fornix and overlying cingulate cortex lesion followed by a Ventromedial Basal Forebrain tissue transplant, n = 9); LBF (lesion followed by a Lateral Basal Forebrain tissue transplant, n = 8); MBF (lesion followed by a transplant of a Mixture of VBF and LBF tissues, n = 8); Lesion Only (lesion and no transplant, n = 8); and Control (sham surgery only, n = 7). An analgesic/antibiotic cream was applied to the sutured scalp after all surgery.

Lesion Surgery.

All surgery was conducted in aseptic conditions under 50 mg/kg Ketamine and 10 mg/kg Xylazine anaesthesia. With the incisor bar set at 2 mm below the interaural line, bilateral radiofrequency lesions of the fimbria-fornix and overlying cingulate cortex were made by maintaining the 0.70 mm diameter tip of the Radionics electrode at 60°C for 1 minute at the following co-ordinates relative to Bregma: cingulate cortex, A -1.3 mm, V -2.0 mm, L ±0.5 mm; fimbria-fornix, A -1.3 mm, V -3.6 mm, L ±0.5 mm and A -1.3 mm, V -3.9 mm, L ±1.6 mm. Control rats received sham surgery but electrodes were not lowered into the brain.

Transplant Surgery.

Transplant surgery, using standard procedures (Bjorklund et al, 1983), was carried out 11 to 15 days after lesion surgery; the non-grafted rats received anaesthetic and scalp incision only. The dam donors were anaesthetised with an overdose of Sodium Pentobarbital on the fourteenth day of gestation. Only foetuses of Crown-Rump Lengths 12/13 mm were used. Both the delay of 11 to 15 days and use of 12/13 mm CRL transplant tissue was intended to maximise the chances of graft survival and acceptance (Dunnett et al, 1989).

On a sterile slide under a dissecting microscope, a coronal section approximately 1-1.5 mm thick was dissected with the rostral cut posterior to the base of the olfactory bulbs, and the caudal cut at the anterior border of the hypothalamus (as shown in figure 1b). This coronal section was then differentially dissected to obtain the VBF and

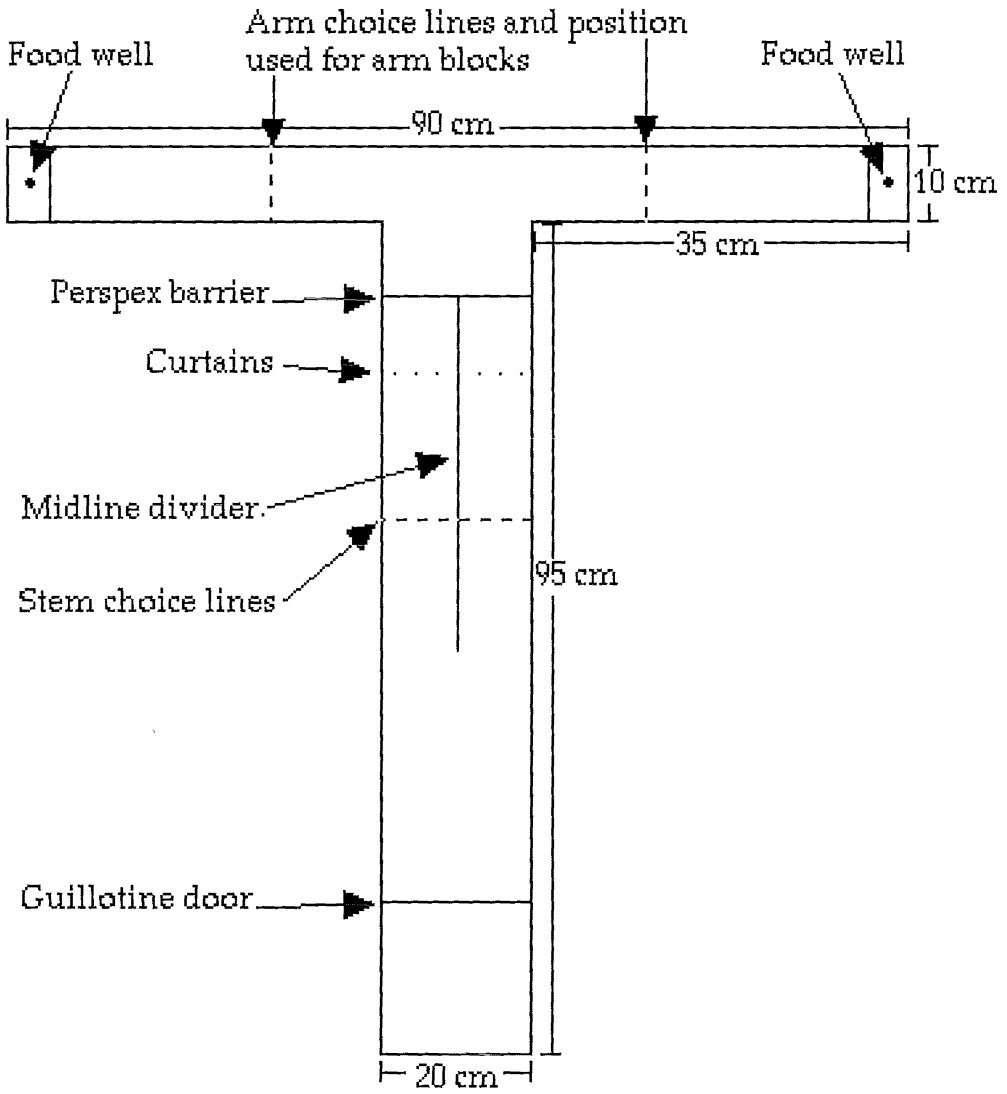


Figure 2. T-maze apparatus.

LBF tissue areas as shown in figure 1g. Pieces of foetal tissue were collected in sterile 0.6% glucose-saline and incubated in 400 µl of 0.1% Trypsin (Sigma, Grade II) glucose-saline solution at 37°C for 20 minutes and then gently rinsed six times in fresh 0.6% glucose-saline. Tissue suspensions were made by mechanical dispersion with a fire-polished Pasteur pipette. Cell suspension viability was assessed with the dead cell

marker Trypan Blue (Sigma) and haemocytometer cell counts. Transplant suspensions had an average viability of 88%, with viability ranging between 96% and 78%, regardless of tissue type. Cell densities were adjusted to between 55,000 to 70,000 viable cells/µl irrespective of the transplant suspension prepared.

Each grafted rat received 1.5 µl intrahippocampal suspension injections at three bilateral sites relative to Lambda: A +4.2 mm, V -3.4 mm, L ±2.2 mm; A +3.5 mm, V -3.5 mm, L ±2.5 mm; A +2.8 mm, V -3.6 mm, L ±3.2 mm. Each graft was injected over 1.5 minutes (a rate of 1 µl / min), and the 10 µl Hamilton syringe was left *in situ* for 2 minutes after delivery of the graft to allow dispersion of the material prior to the gentle removal of the syringe. Two rats additional to the group numbers mentioned previously were lost under anaesthetic during these procedures.

Behavioural Tests.

Preoperative T-maze testing. The T-maze apparatus and procedure was based on that of Helper et al (1985) and comprised a stem discrimination (reference memory) and an arm discrimination (working memory). See figure 2 for a diagram of the main features of each T-maze. Two identical T-mazes were constructed of painted wood, with a 2 cm wood border, and elevated 88 cm above the floor. Each arm of the T-maze was 10 cm wide, and 35 cm long, and had at its end a 1.5x1.5 cm food well drilled in the centre of a 10x4 cm piece of wood. The stem was 20 cm wide and 95 cm long and comprised a 28 cm long start area separated from the remaining stem by a 6 cm wide guillotine door. Dividing the stem along the midline was a 12 cm high, 31.5 cm long piece of wood, near the end of which a perspex barrier could be positioned at either side as needed. White opaque

curtains hung from a frame on either side of the midline divider, 7.5 cm in front of the perspex barrier, thus obscuring the barrier and the arm choice point at the end of the stem from the advancing rat.

The mazes were positioned close to one another (arms 50 cm apart) with stems orientated at an angle of approximately 60° to one another - this arrangement being maintained throughout testing and in both pre-operative and post-operative testing rooms. Both testing rooms were well lit and contained many extra-maze cues. To control for rats attending to intra-maze cues in either particular maze, since there is evidence that rats are more likely to attend to these than extra-maze cues (Kesner, 1993), the mazes were randomly alternated approximately every three or four testing sessions. However, for any given rat in any given maze location, the side on which the perspex barrier in the stem was positioned remained constant throughout testing.

After initial familiarisation to run both arms in both mazes, including adaptation to curtains and barriers, the rats were tested for spatial reference and working memory by being trained to negotiate the stem barriers and run to the end of an arm for a 0.1 g piece of chocolate. For each rat a testing session consisted of six trials, three in each of the two maze positions. Each trial consisted of a double run in the same maze - a forced run in which the rat was directed to one arm by virtue of the second arm being blocked, followed by a choice run in which the rat was free to choose either arm. During this double run trial, the rat performed the reference stem task twice (once for each run). Thus, in a session of 6 trials the maximum correct stem discrimination score was 12, and the maximum correct arm discrimination score was 6.

All rats were run in a home-cage squad of four and the interval between each double run was 3 to 4 minutes during which time the rat was returned to a separate holding home cage while the other rats in that squad were run.

The stem discrimination component of this T-maze procedure is a test of reference memory because it requires the learning of a rule which is invariant over trials. For half the rats, balanced across groups, the perspex barrier in the stem was always placed on the left side of the stem for the maze located on one side of the room, and always on the right side of the stem for the maze located on the other side of the room. So for these rats correct reference memory performance would mean taking the right stem side on the first maze and the left stem side on the second. Opposite positions of the barriers were used for the other half of the rats. Choice lines were marked 10 cm beyond the start of the central stem partition (see figure 2); a choice was recorded when the rat placed its head and front paws over this line. If the rat made the wrong stem choice it was free to return to the start of the stem, take the alternative path and then proceed to the arm task.

The arm discrimination component of the T-maze was a test of working memory because it required trial-dependent alternation. At the start of each trial a 0.1 g piece of chocolate reward was placed in the food well at the end of each arm. First the rat was given a forced run, or 'information run', where a large wooden block stopped entry into one of the arms. The rat was forced to enter one arm and would eat the food reward. The rat was then returned to the start area, the wooden block removed, and after 3 to 4 seconds delay the guillotine door was opened and the rat entered the maze for the choice run. If the rat entered the arm that had been blocked on the forced run a correct arm response was recorded and the rat

received the other food reward at the end of that arm. If the rat chose the arm that had been unblocked on the forced run an incorrect response was recorded, and no food reward was available. Again, head and front paws over a line 10 cm down the length of the arms delimited when a response had been made (shown on figure 2).

Fellows (1967) sequences were used to arrive at a balanced distribution of unblocked arms in the forced runs, and to determine which particular maze on which the two runs of each trial were conducted. These sequences ensure that the rat cannot used unwanted alternation strategies to attain high scores and must attend to extra-maze cues to solve the task.

All animals were tested on a 'blind' basis and received between 22 and 34 sessions of preoperative T-maze training up to a criteria score of >60 from a possible 72 (that is >83% correct) in the last 6 stem task sessions, and a score of ≥33, from a possible 36 (that is ≥92% correct) in the last 6 arm task sessions. Two rats in addition to the group numbers mentioned previously failed to reach these criteria and were not used further. From the last 18 preoperative sessions total scores for both the stem and arm tasks were calculated for each rat and these used to form a ranked list. Each ranked subject was taken in order and randomly assigned to an experimental group.

Postoperative T-maze Testing. Eighteen days after completion of transplant surgery all rats were again tested on the same T-maze apparatus in the same manner for a further 40 sessions in a new testing room. The use of a new testing room was to investigate whether a reference memory rule learnt through attendance to one set of extra-maze cues could be reacquired in a new room where the extra-maze cues would

be different and only the orientation of the mazes to each other would remain the same. It is known that rats without preoperative training and fimbria-fornix lesions cannot learn such a reference memory rule (Dalrymple-Alford, 1994).

DRL Testing. The rats were trained and tested in 10 identical operant chambers, each under control of a separate Rockwell microcomputer (Microcomputer Control Systems, Minneapolis). The interior dimensions of each operant box were 30.5 cm wide, 25 cm deep, and 27.5 cm high. Ten cm from the floor a 3 cm wide lever extended 2.5 cm into the chamber. A light 4 cm above the lever was lit during the session.

Two days after the end of postoperative T-maze testing, the rats were habituated to the chambers, shaped to bar press, and then placed on a continuous reinforcement (CRF) schedule for seven daily 40 minute sessions. All rats made in excess of 200 responses per session by the second or third day of CRF training. Rats then received 40 daily 40 minute DRL-20 sessions.

The times between one bar press response and the next, the Inter-Response Times (IRTs), were examined for all experimental groups. Modal IRTs were found to be considerably lower than 20 s, particularly for transplant and lesion-only groups which showed little or no improvement over sessions, indicating that a waiting period of this length was too difficult for non-controls (IRT modes are shown in figure 3). In an attempt to make the task easier, and more sensitive to any potential improvement in performance, the waiting period was then reduced and all rats were placed on a DRL-10 schedule for a further 40 sessions.

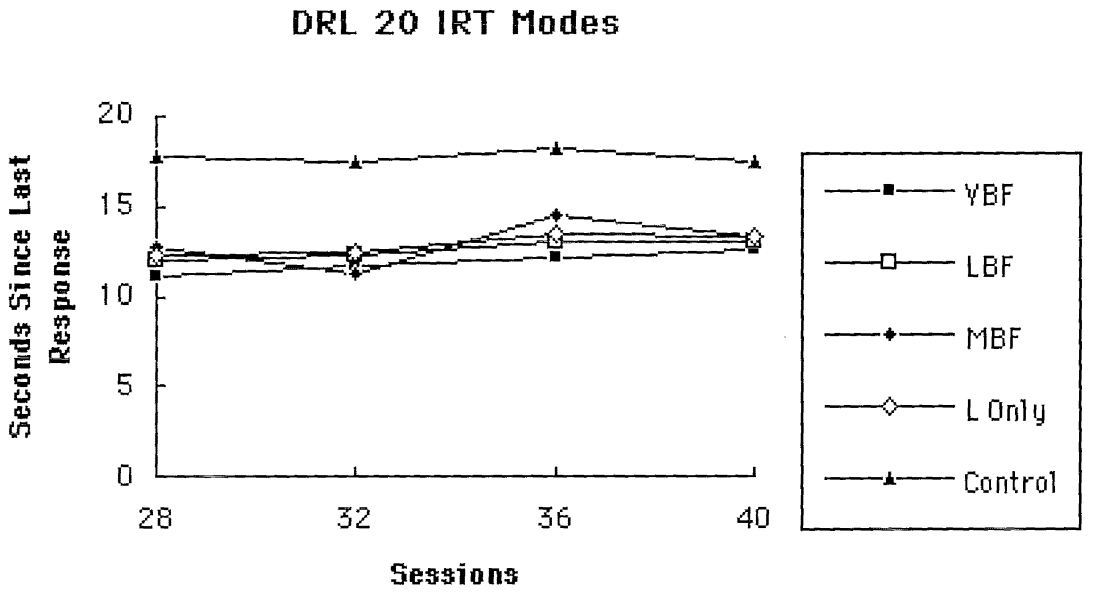


Figure 3. IRT modes for DRL-20 task.

Activity Measures. A week after completion of DRL testing spontaneous locomotor activity was monitored using two identical activity boxes constructed of clear perspex (30 by 40 cm wide, by 18 cm high; as per Dunnett et al, 1989), each with a sawdust floor and a wire lid. The testing environment was a quiet, dimly lit room (5 lx in the activity boxes). Two photo-electric beams were mounted on the 40 cm face, 1 cm from the floor, and 8 cm from the ends of each box. A Rockwell microcomputer (Microcomputer Control Systems, Minneapolis) was used to concurrently record two separate measures of activity: the total number of beam breaks, and 'crossings'. Crossings were measured as per Cassel et al (1991) and occurred when an animal proceeded directly from breaking beam A to breaking beam B, or vice versa. Crossings and total activity were recorded

in 10 minute bins in a single 60 minute session for each rat, using clean sawdust for each rat.

Histology.

Staining. At the end of testing, rats received an overdose of Sodium Pentobarbital and were perfused intracardially with 0.9% saline followed by 4% formalin. The brains were postfixed in 4% formalin then stored in a 30% sucrose-formalin solution at 4°C until sectioning. Frozen 50 μ coronal sections were made through the septo-hippocampal extent. Every fourth section was stained for AChE activity by the thiocholine method, with 0.01% ethopropazine as an inhibitor of non-specific esterases and 0.25% silver nitrate to enhance the sulphide reaction product. Adjacent sections were stained for Nissl substance with Cresyl Violet.

Ratings of AChE positivity. For all groups that received a lesion separate subjective estimates of AChE-positivity was made for the hippocampal Ammon's Horn (CA1, CA2, and CA3) and the dentate gyrus (including CA4 and hilus areas) for the right and left hippocampus. Such ratings were performed at one coronal location per brain at an anteriority where the habenular nucleus appeared most prominent; A -3.80 relative to Bregma (Paxinos and Watson, 1986). Figure 4 shows the relevant hippocampal features at this anteriority.

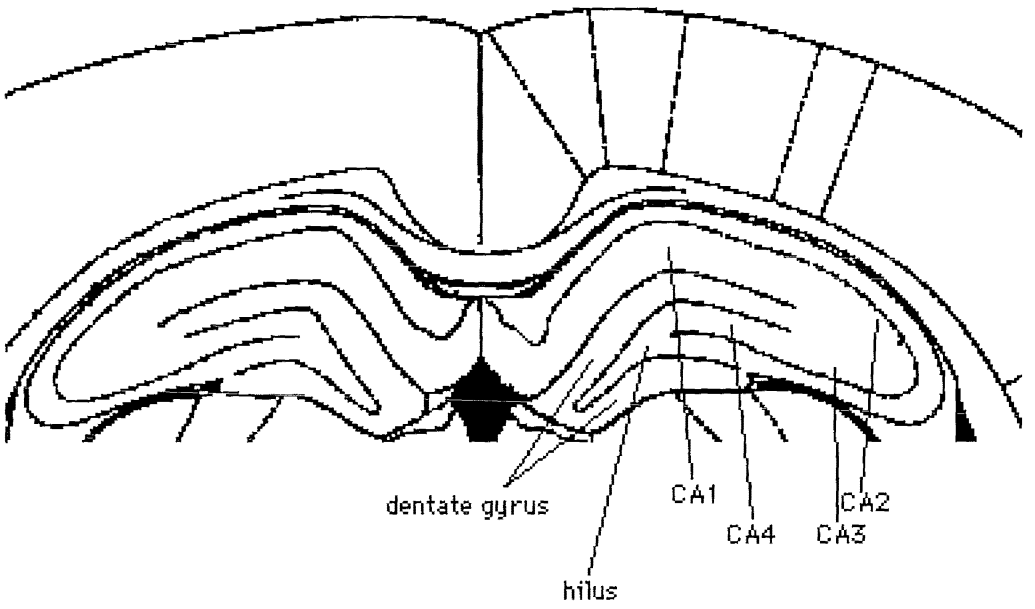


Figure 4. Main hippocampal areas.

For intact sections of control brains, a rating score of 10 was given for the AChE-positivity of the hippocampal Ammon's Horn and for the intact dentate gyrus region. These control scores were then used as standards against which the corresponding regions of other brains were compared. In some instances, the AChE-positivity was apparently greater than normal, so a rating scale of 0-12 was used. These measures were expressed as a percent of normal AChE-positivity and averaged across the left and right hippocampal formation for both Ammon's Horn, and dentate gyrus regions. It was also noted whether the ventral hippocampus had 'similar', 'less' or 'more' AChE-positivity compared to dorsal positivity at approximately A -4.80 relative to Bregma.

Results.

Behavioural results.

Working Memory. Arm task performance over preoperative and postoperative T-maze sessions is shown in figure 5.

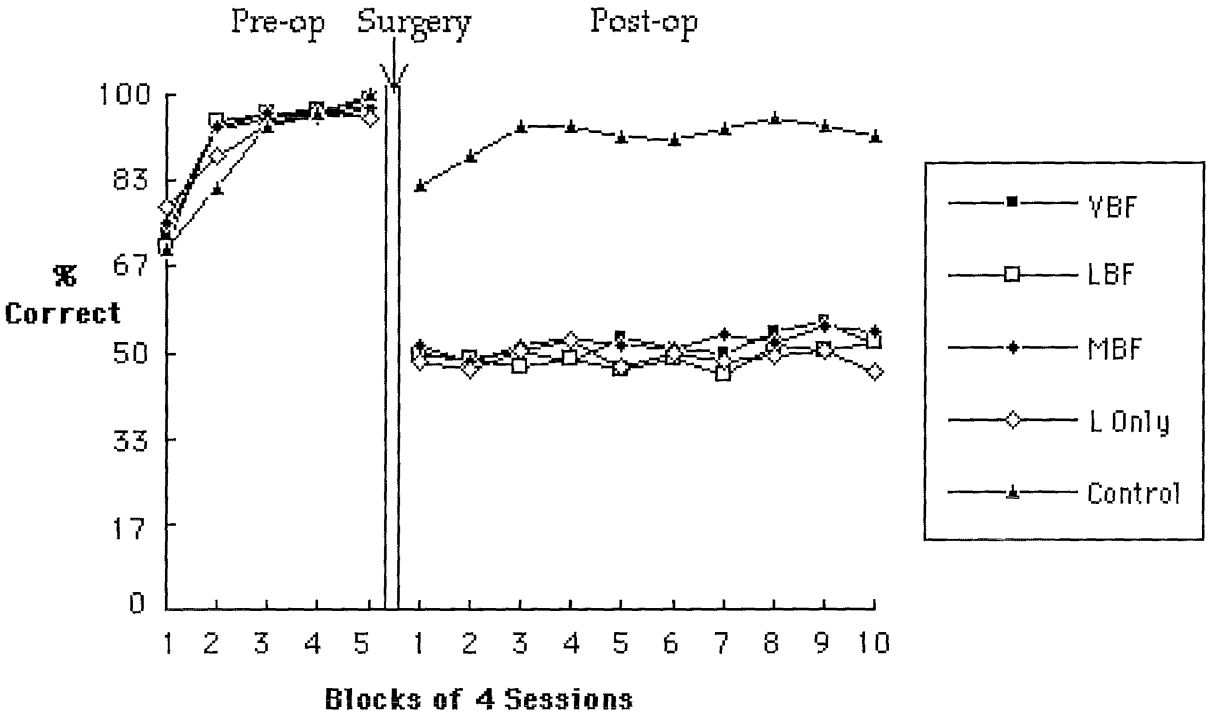


Figure 5. Working memory performance for all sessions.

The 20 most recent preoperative sessions were blocked by fours in a 5(groups) x 5(repeated measures of preoperative session blocks) analysis of variance. All preoperative groups quickly learnt the arm task, and by the third preoperative session block were attaining near perfect scores, an increase which was significant across sessions ($F(4,35) = 52.29$, $p < 0.0001$).

As desired, ranked random assignment to groups resulted in arm task scores for preoperative groups which were not significantly different from each other ($F(4,35) < 1.0$).

Postoperatively, only controls were capable of the task at near preoperative levels, while all groups which received a lesion performed at chance throughout the 40 postoperative sessions. This difference in postoperative performance produced a significant groups effect in the 5(groups) x 10(repeated measures of postoperative session blocks) analysis of variance, $F(4,35) = 47.92$, $p < 0.0001$. While one control rat performed poorly at the arm task postoperatively, dragging down the average for controls in figure 5, this rat still scored better than all but two lesion-only rats. Analysis also revealed a significant sessions effect ($F(9,35) = 2.99$, $p < 0.005$) as group scores increased over sessions, particularly control scores in the first three session blocks, but this increase was not sufficient to produce an interaction effect ($F(36,315) = 1.11$, $p < 0.31$). No transplant amelioration of the lesion induced working memory deficit was present, neither was any transplant group different from any other (as confirmed by Newman-Keuls pairwise comparisons). Transplant groups were also not significantly different from lesion-only rats.

Reference Memory. Preoperative and postoperative stem task performance is shown in figure 6.1.

Preoperatively, all groups increased in ability at the stem task up to near perfect scores by the last session block prior to surgery resulting in a significant sessions effect in the 5(groups) x 5(repeated measures of preoperative session blocks) analysis of variance ($F(4,35) = 71.58$, $p <$

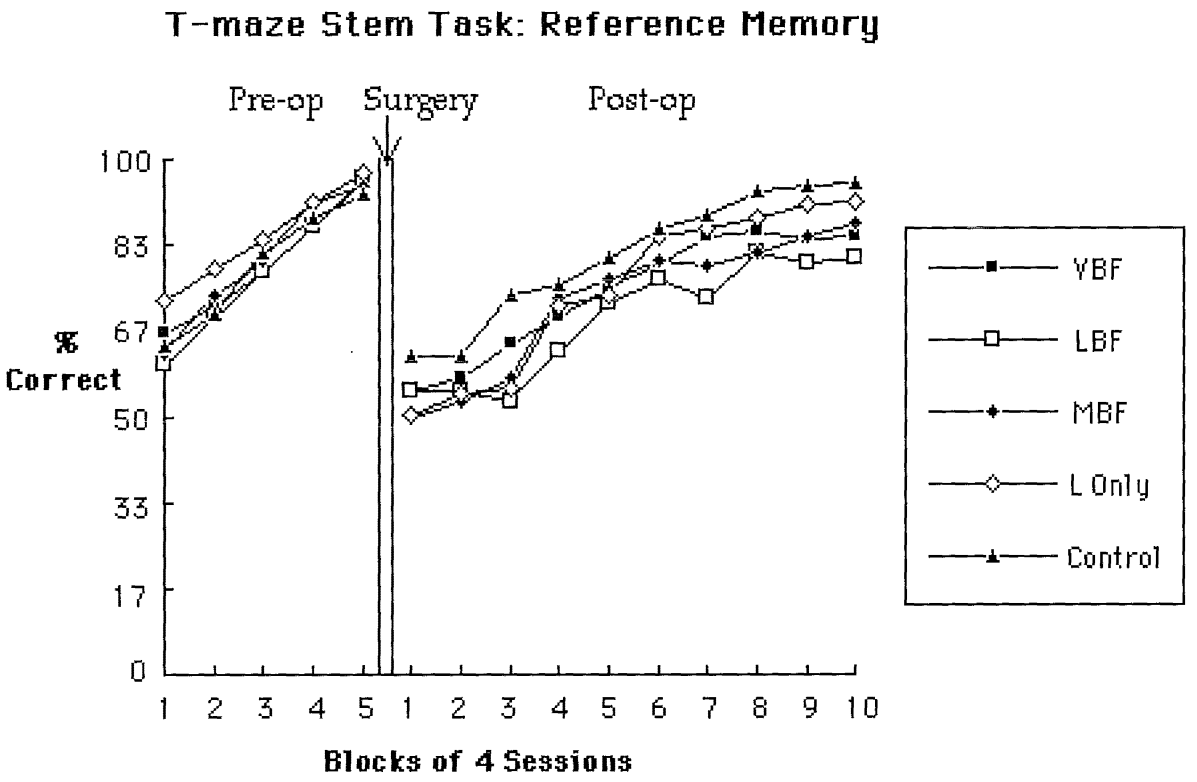


Figure 6.1. Reference memory performance over all sessions.

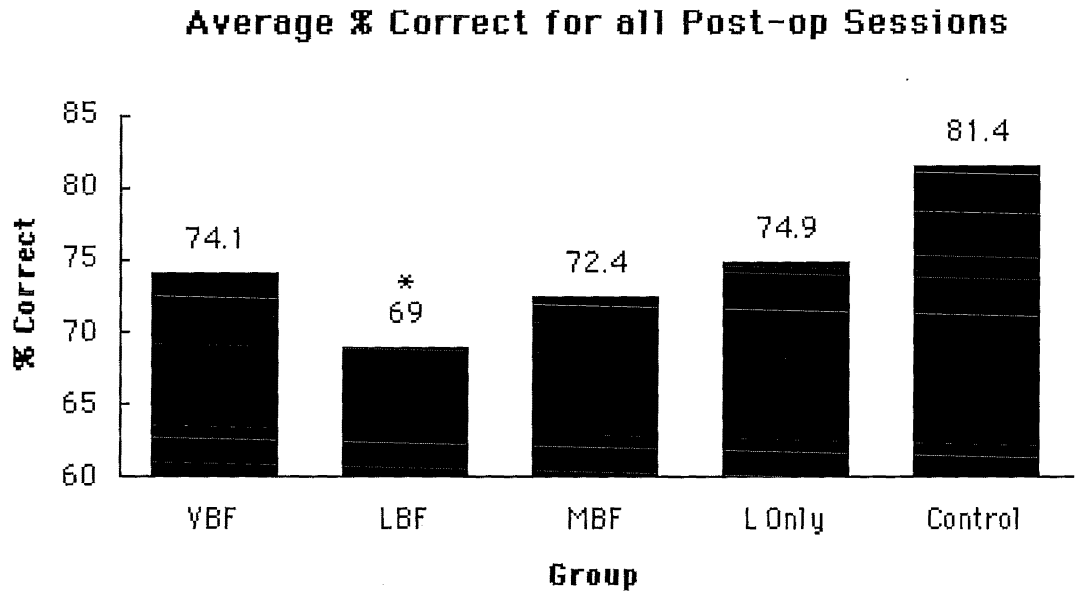


Figure 6.2. Average % correct for each group over reference memory sessions. * Group LBF was significantly different to the control group ($p < 0.05$, Newman-Keuls test). No other significant differences were found.

0.0001). As desired, the five groups did not significantly differ before surgery treatments ($F(4,35) < 1.0$).

Postoperative stem task performance for all groups dropped to chance level in the new testing room, before recovering again resulting in a significant sessions effect ($F(9,35) = 2.99$, $p < 0.005$) in the 5(groups) x 10(repeated measures of postoperative session blocks) analysis of variance. As the performance level of the first two session blocks was at chance for all groups, they have re-learnt the reference memory task by apparently attending to new extra-maze cues in the new testing room. However, the recovery in postoperative responding was not complete, as a comparison of the last preoperative session block with the last postoperative session block demonstrated. Postoperative responding remained significantly lower than preoperative responding as shown in a significant session block effect in the 5(group) x 2(session blocks) analysis of variance, $F(1,36) = 16.55$, $p < 0.002$. No significant group or interaction effect was present (group: $F(4,36) = 1.93$, $p < 0.127$; group x session block: $F(4,36) = 2.59$, $p < 0.053$).

The analysis of variance also revealed a significant group main effect ($F(4,35) = 2.68$, $p < 0.05$). Newman-Keuls pairwise comparisons revealed that transplant group LBF scored significantly more poorly at postoperative reference memory than did the control group, but was not significantly different from any of the other groups which showed intermediate levels of performance between high scoring controls and low scoring LBFs (these differences are shown in figure 6.2). As lesion-only rats are not significantly impaired relative to controls, and LBFs are not significantly impaired relative to lesion-only rats, the finding that LBF's performance is significantly different from controls cannot be attributed to LBF tissue per se. As such it was the combination of a lesion

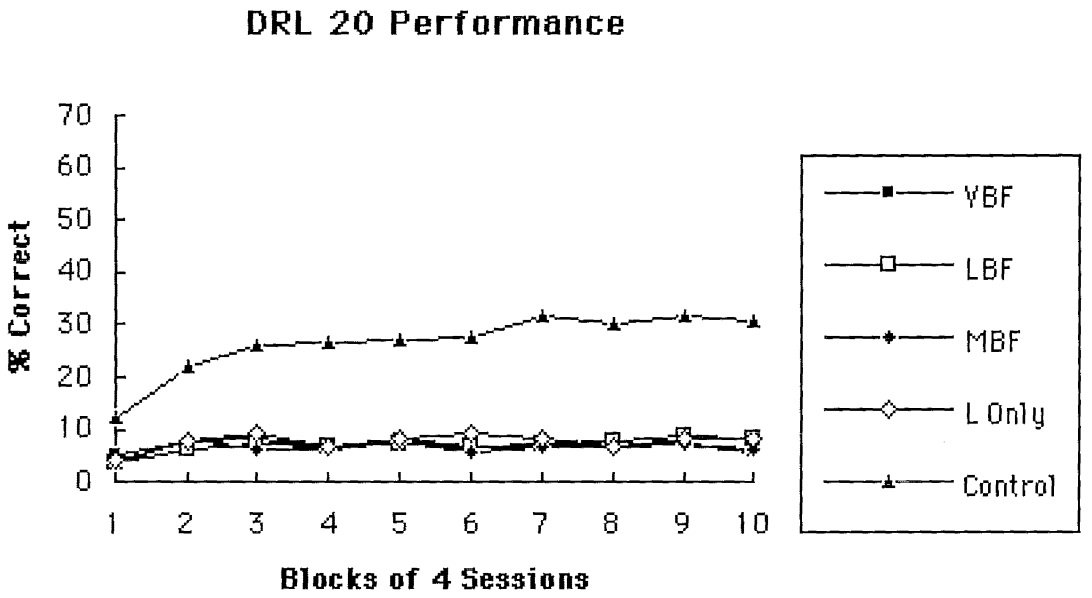


Figure 7. DRL-20 performance over all sessions.

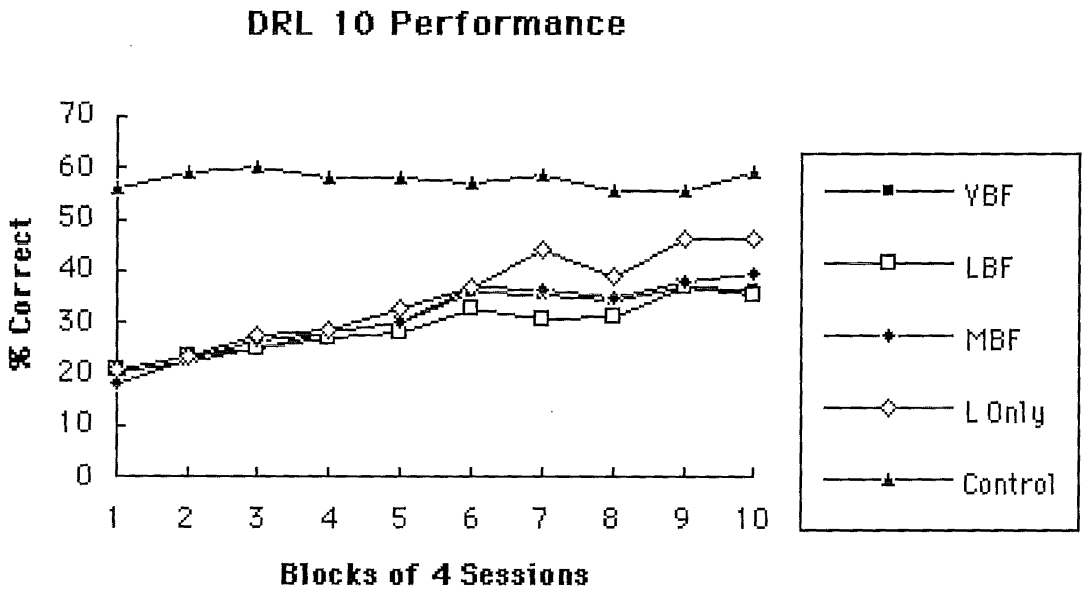


Figure 8. DRL-10 performance over all sessions.

followed by an LBF transplant that had a significantly deleterious effect on the reacquisition of reference memory.

DRL-20 Testing. Figure 7 shows DRL-20 performance over the 10 session blocks. Only controls achieved any level of proficiency at the DRL-20 task, that of 30% correct, while all other groups scored no more than 5% correct. Control scores increased significantly over sessions and were significantly different from all other groups, resulting in significant group, session, and interaction effects in the 5(groups) x 10(repeated measures of session blocks) analysis of variance (groups: $F(4,35) = 21.05$, $p < 0.0001$; sessions: $F(9,35) = 9.27$, $p < 0.0001$; groups x sessions: $F(36,315) = 2.82$, $p < 0.0001$). Simple effect analysis showed that scores for all non-control did not change significantly over sessions.

Analysis of the Inter-Response Times (IRTs; the time elapsed between one bar press made by the rat and the next) for the DRL-20 task showed modal IRTs to be much lower than the 20 second waiting period required, especially in non-control groups. Figure 3 shows IRT modes over sessions for each group, and explains the poor performance levels shown in figure 7.

DRL-10 Testing. Figure 8 shows DRL-10 performance over the 10 session blocks. Controls averaged approximately 60% correct over all session blocks, significantly higher than all other groups in the 5(groups) x 10(repeated measures of session blocks) analysis of variance (groups: $F(4,35) = 6.92$, $p < 0.0005$). All groups, particularly the lesion-only group, showed improved performance over sessions, and this resulted in a

significant sessions and interaction effect (sessions: $F(9,35) = 25.19$, $p < 0.0001$; groups x sessions $F(36,315) = 2.42$, $p < 0.0001$). Simple effects confirmed that the performance of the control group did not change significantly over sessions and that the scores for lesion groups did increase significantly over training on the DRL task.

As lesion-only rats averaged higher than transplant rats in the last four session blocks, and simple main effects showed a group effect at session block 9 which only narrowly passed significance ($F(4,55) = 2.56$, $p = 0.049$), individual session block contrasts were carried out to more closely investigate the relationship of the lesion-only group to other groups[1]. It was found that up to and including session block 6 the lesion-only group was significantly different from controls ($p \leq 0.004$), but in the last four session blocks the lesion-only group failed to be significantly different from controls ($0.22 \geq p \geq 0.051$). However, at no time was the lesion-only group significantly different from other transplant groups ($p \geq 0.1$). Neither did any transplant group ever fail to be significantly different from controls ($p \leq 0.02$). After starting off as significantly different from controls the performance of the lesion-only group had become intermediate between controls and transplant groups by the last four session blocks as it was not significantly different from either, while all transplant groups remained significantly different from controls at all times.

[1] See appendix for a table of F, p and df values for contrasts performed.

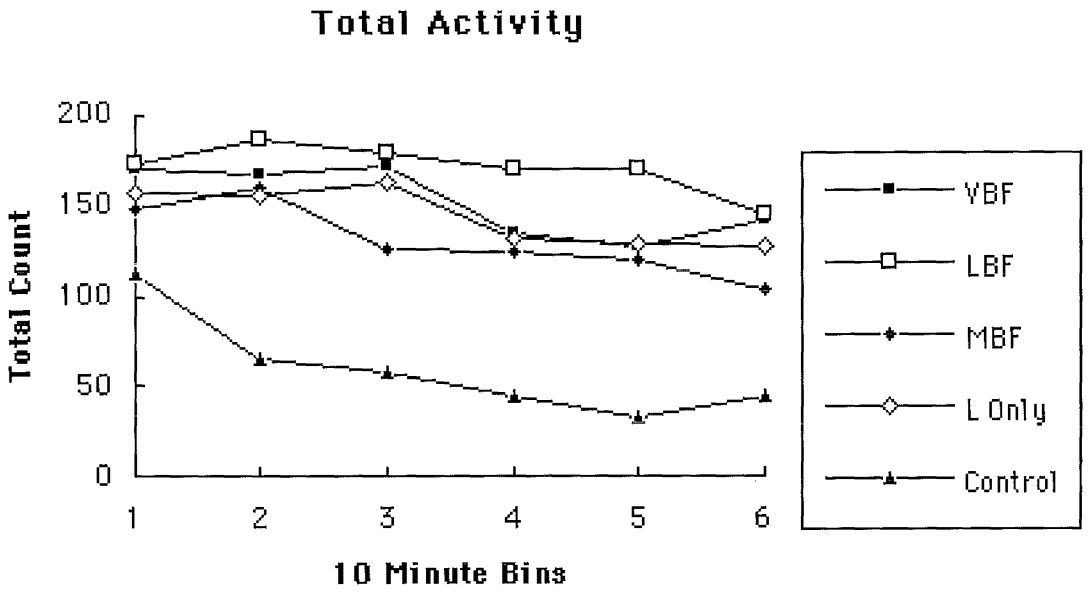


Figure 9. Activity as measured by total beam breaks.

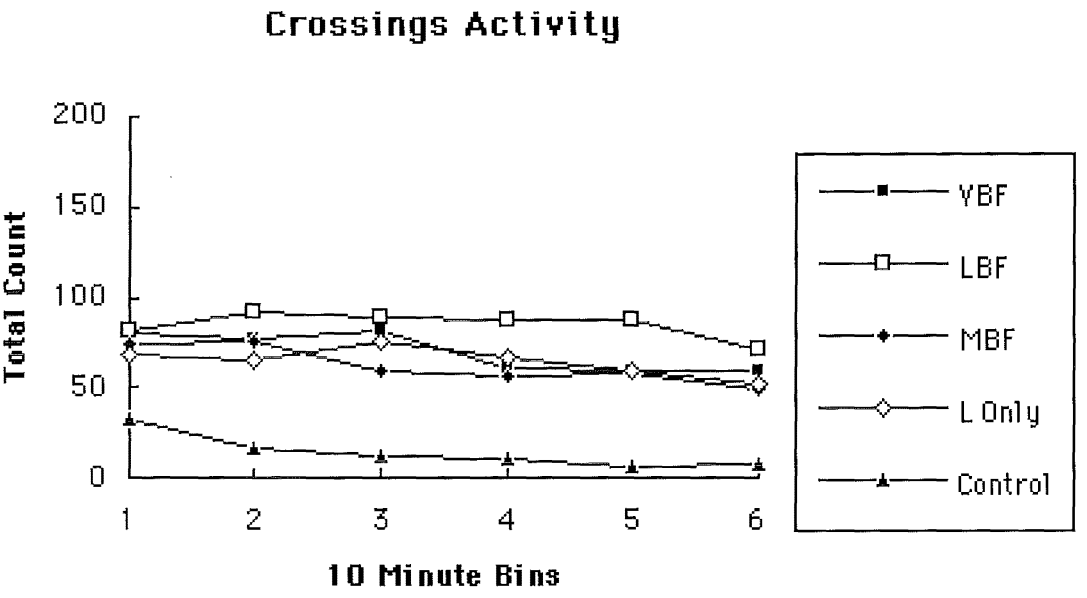


Figure 10. Activity as measured by "crossings" between beams.

Activity Measures. Two measures of activity were used: total beam breaks, and crossing, for which scores over sessions are shown in figures 9 and 10 respectively.

Total beam breaks showed significant group and bins effects in the 5(groups) x 6(repeated measures of 10 minute bins) analysis of variance. Controls were less active than lesion groups (groups: $F(4,35) = 6.36$, $p < 0.001$), and there was no transplant effect evident in activity as confirmed by Newman-Keuls pairwise comparisons which demonstrated lesion groups as not significantly different from each other. Activity for all groups decreased over bins as would be expected due to habituation to the chambers (bins: $F(5,35) = 14.02$, $p < 0.0001$).

Crossings analysis, a 5(groups) x 6(repeated measures of 10 minute bins) analysis of variance, found identical results to those found in the analysis of total beam breaks: controls were significantly less active compared to all other groups ($F(4,35) = 7.98$, $p < 0.0005$), and activity for all groups decreased significantly over bins ($F(5,35) = 7.56$, $p < 0.0001$). As in the total beam breaks activity measure no transplant ameliorated decrease in activity was evident as Newman-Keuls pairwise comparisons revealed all transplant groups to be not significantly different from each other or from lesion-only rats.

Histology.

Nissl-stained sections showed complete ablation of the fimbria-fornix and overlying cingulate cortex in all rats with lesions. In many cases some additional damage to the caudate putamen was evident, but typically the dorsal surface of the thalamus was free of damage. Nissl-stained sections

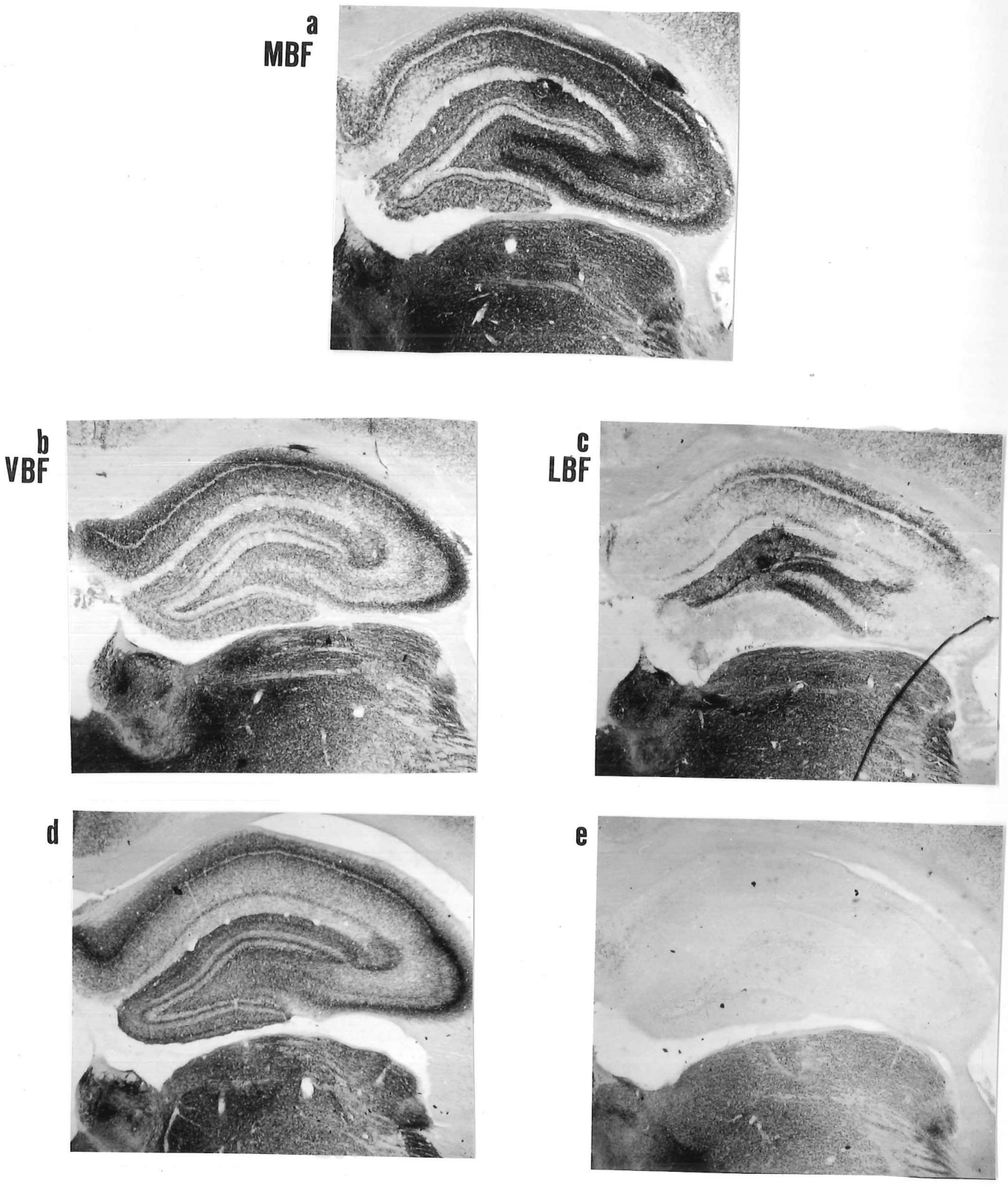


Figure 11. Representative photomicrographs from each experimental group.

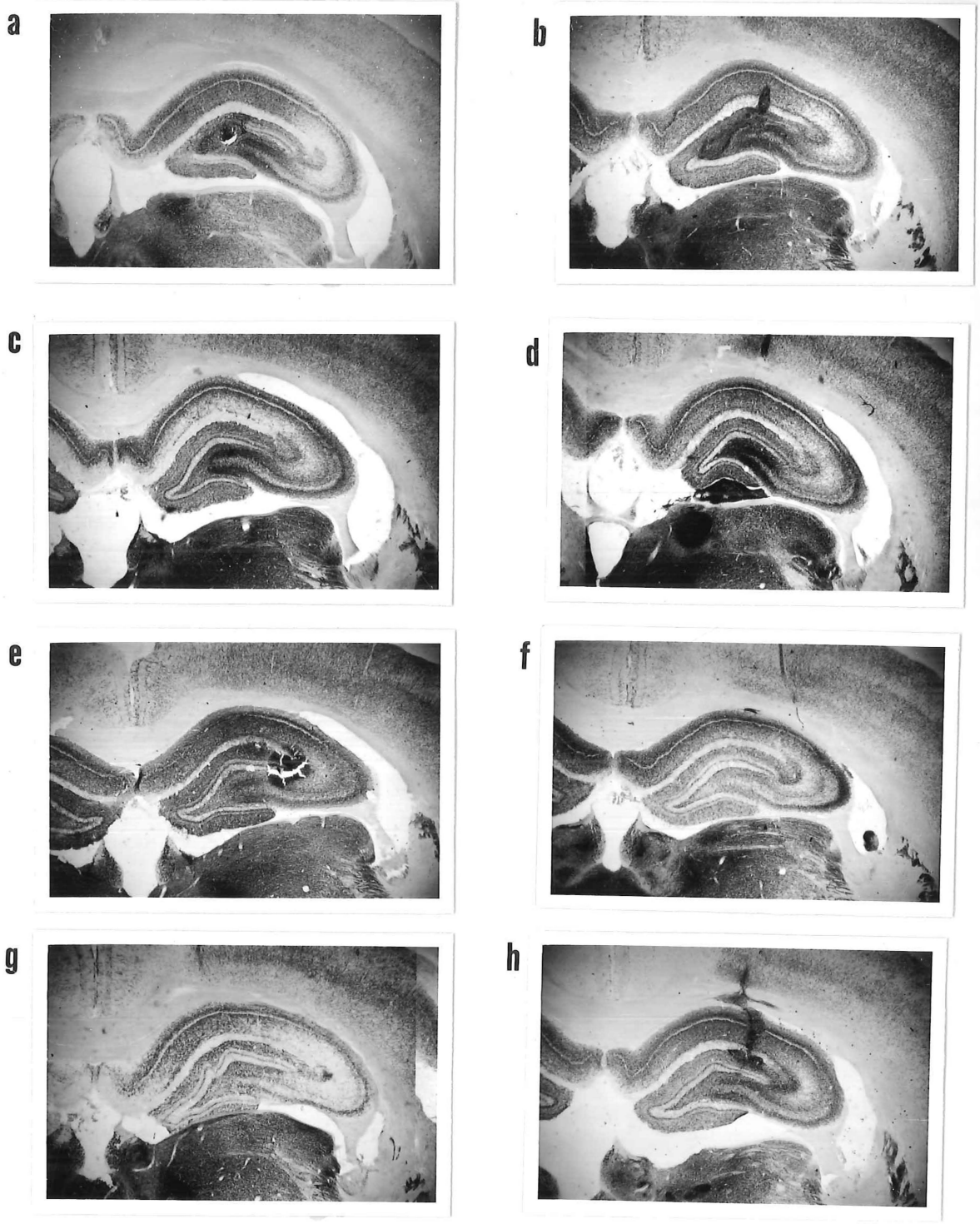


Figure 12. Group VBF.

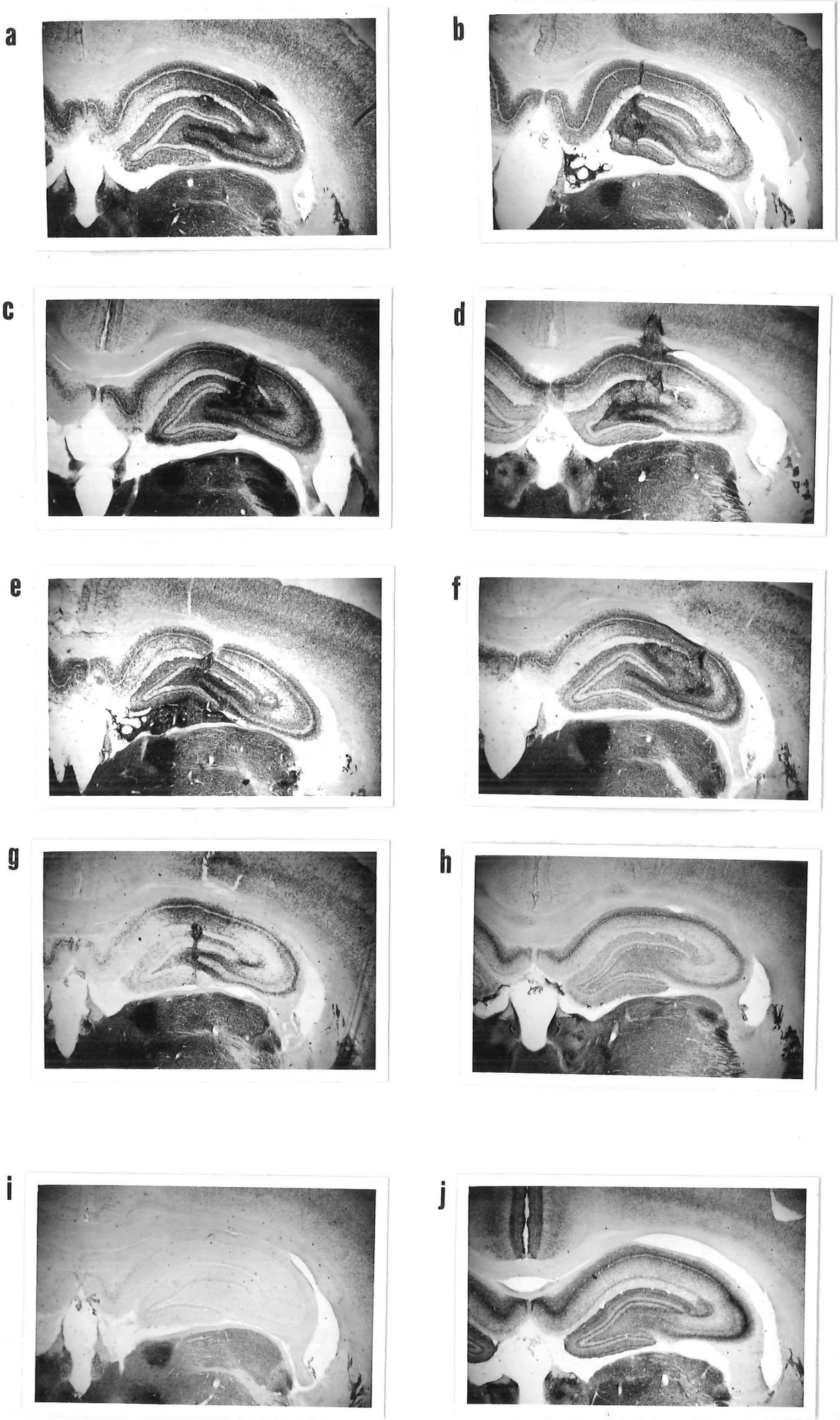


Figure 13. Group MBF.

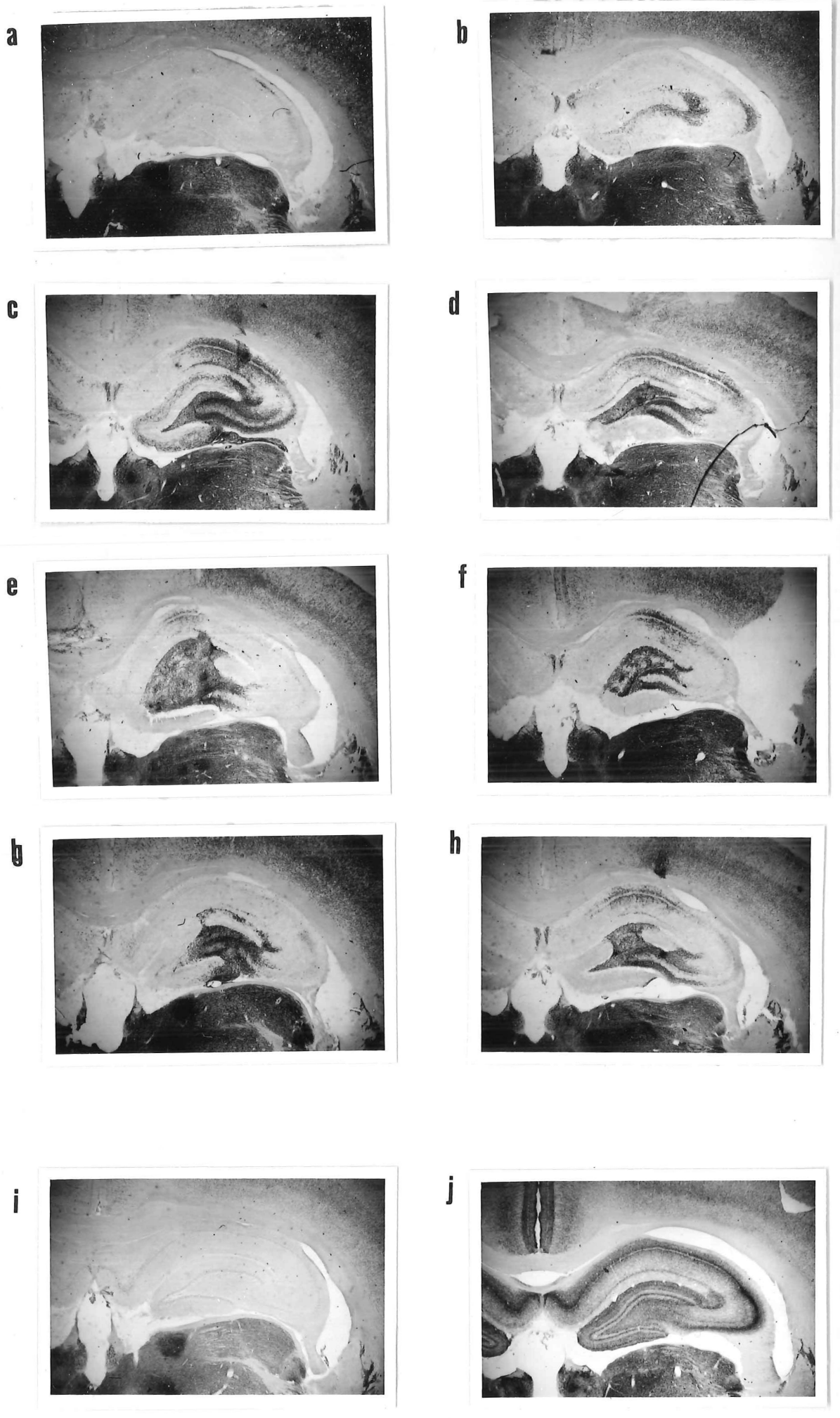


Figure 14. Group LBF.

also showed glial cells centrally clustered in the body of transplant tissue which was observed in most brains, but not exclusively in any particular groups.

Figure 11 shows a representative photomicrograph from a rat in each experimental group of the coronal section at anteriority A -3.80 relative to Bregma where AChE positivity ratings were made. The morphological characteristics of each group are distinct and consistent, showing bilateral survival of grafts in all transplant rats. Figure 11e shows the AChE-positivity of the dorsal hippocampus of a lesion-only rat, showing complete loss of AChE-positive fibres, while figure 11d shows a representative control showing normal AChE innervation. Figures 11e and 11d can be contrasted with 11a, 11b and 11c which show corresponding representative sections from groups MBF, VBF, and LBF respectively.

Figures 12, 13 and 14, respectively show, photomicrographs of each member of each transplant group VBF, MBF and LBF, to demonstrate the consistency and distinctiveness of the reinnervation pattern with each kind of tissue. At the bottom of each page is shown a small version of the representative lesion-only and control photos shown in figure 11 as way of contrast.

VBF grafts. Graft group VBF (figure 12) yielded AChE-positive reinnervation which most closely resembled the normal innervation pattern. Overall, all fields of the hippocampus were reinnervated in a manner largely reminiscent of the normal laminar patterns evident in intact rats. In three VBF group members (figures 12b, h and i) darkly stained, but very small and minimally disruptive transplant masses

existed inside the hippocampus in the injection tracks. VBF group member 12h showed leakage of transplant material up the injection tracks into the overlying cortex, but again with no disruption of surrounding structures. Compared to controls slight hyperinnervation of the dorsal edge of the CA1 field was evident in most VBF hippocampi. Weak staining of the CA3 field was also evident compared to controls in three VBF group members (figures 12a, b and g). Only one VBF hippocampus showed a transplant mass in the cavity between the ventral surface of the dentate gyrus and the thalamus (figure 12e). The hippocampi in all VBF rats were better reinnervated than any LBF hippocampus.

LBF grafts. Reinnervation with LBF tissue (see figure 13) appeared to be restricted entirely to the transplant mass itself and while transplant masses were well stained, overall reinnervation was very poor and patchy. In at least three LBF group members dark AChE stained areas were interspersed with blank poorly reinnervated areas (figures 13c, e and f). Most reinnervation that did take place with LBF tissue occurred in the hilus and dentate gyrus areas, although in many hippocampi additional partial reinnervation occurred in the CA1 field (figures 13c, d, e, f, g and h). Other than reinnervation of dentate gyrus, hilar, and CA1 areas reinnervation was strikingly poor or absent. The most poorly reinnervated LBF subject (figure 13a) appeared almost indistinguishable from a lesion-only hippocampus, showing only the smallest wisp of reinnervation in the dorsal edge of the CA2 field. Laminar patterning in reinnervated areas was fragmented and in at least one subject (figure 13e) entirely disrupted in the dentate gyrus and hilas areas. However only one group member showed a small transplant mass that extended outside the

hippocampus (figure 13c), lodging in the cavity between the ventral CA3 field and the thalamus.

MBF grafts. While MBF group members (see figure 14) were generally well reinnervated laminar disruption and blank patches were also present. At least two subjects showed disruption of laminar patterning in the reinnervated hilas and dentate gyrus areas (figures 14b and d), while another showed disruption between the CA1 and CA3 fields (figure 14f). Darkly stained transplant masses were present in three subjects around injection tracks (figures 14c, d and g), and two hippocampi showed transplant masses which extended into the ventro-medial ventricle (figures 14b and e). Qualitatively MBF group members appear more similar to members of the VBF group than the LBF group by virtue of the fact that generally MBF reinnervation is good, while LBF reinnervation is very poor. However, in some senses MBF transplants show combination effects of the constituent tissues VBF and LBF. MBF hippocampi show the patchiness of reinnervation of LBF grafts, while also showing the generally good levels of AChE-positive staining throughout all fields of the hippocampus present in VBF grafts.

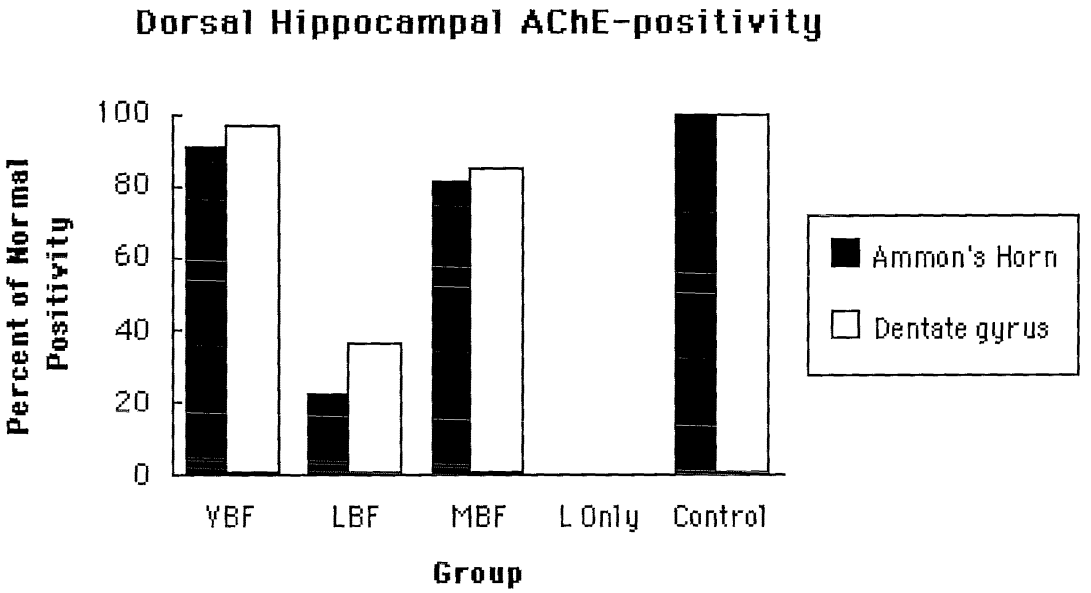


Figure 15. Hippocampal reinnervation as measured by AChE-positivity.

AChE-positivity Ratings. Figure 15 shows positivity scores for all groups averaged bilaterally for hippocampal locations Ammon's Horn and dentate gyrus. Moderate to excellent cholinergic reinnervation was present in groups MBF and VBF (averaging Ammon's Horn and dentate gyrus scores gives 83% and 93% of normal innervation for the two groups respectively). By contrast, poor reinnervation was present in group LBF which showed little AChE-positivity outside the graft tissue, although the graft itself was densely stained for AChE (scoring 29% of normal over Ammon's Horn and dentate gyrus aspects).

All lesion groups scored zero AChE-positivity for the dorsal hippocampus, and as such were not suitable for statistical analysis since the scores contained no variance. Similarly, the control group was not included in the analysis since all control scores were 10. Hence statistical analysis comprised only the three transplant groups in a 3(groups) x

2(hippocampus location) analysis of variance. A significant groups and location effect were found (groups: $F(2,22) = 39.28$, $p < 0.0001$; location: $F(1,22) = 6.23$, $p < 0.05$). Newman-Keuls pairwise comparisons of the groups effect revealed groups MBF and VBF as not significantly different from each other in terms of AChE positivity, while both MBF and VBF were significantly different to group LBF. This result confirms the qualitative impression gained from slides that MBF group members appeared more similar to member of the VBF group than the LBF group. Newman-Keuls pairwise comparisons of the significant location effect revealed that positivity in the Ammon's Horn region was significantly lower than that in the dentate gyrus for all transplant groups, an effect especially pronounced in the LBF group. This result confirms qualitative impressions of LBF slides that the hilus and dentate gyrus areas appeared more darkly stained.

The descriptions of positivity in the ventral aspect of hippocampi as either "less", "similar", or "more" AChE-positive than the dorsal aspect revealed a similar pattern as compared with the analysis of AChE-positive ratings: VBF and MBF groups were alike, and showed ventral AChE-positivity mostly similar to their dorsal positivity, while group LBF showed mixed subjective descriptions of "less", "similar" and "more" ventral positivity compared to dorsal positivity. Lesion only hippocampi, as would be expected in the absence of a transplant and the presence of a highly effective lesion, showed "more" ventral positivity than dorsal positivity (Dalrymple-Alford, 1994).

Discussion.

Transplant morphology and reinnervation.

AChE histology confirmed bilateral survival of implanted tissue, and showed remarkable differential reinnervation across transplant groups. Averaging Ammon's Horn and dentate gyrus hippocampal AChE-positivity scores (figure 15) to consider the overall reinnervation for each group, groups VBF and MBF showed excellent cholinergic reinnervation at a mean of 93% and 83% respectively of normal levels, while transplant group LBF showed poor reinnervation at 29% of the normal level. The low reinnervation score given hippocampi which received LBF tissue is not due to LBF tissue's lack of cholinergic cell content, as LBF tissue is richly cholinergic, it is due to the fact that LBF tissue does not reinnervate to any extent outside the mass of transplant material, although the mass itself is strongly AChE-positive. LBF tissue exclusively contains NBM cells, but since NBM cells project to the cortex not the hippocampus, transplantation of this material to the hippocampus shows poor reinnervation because of this cellular association (Dunnett et al, 1986). NBM cells do not reinnervate the hippocampus effectively because the neurochemical environment is "inappropriate" for this type of tissue; a process that presumably involves cell-cell interaction and recognition, or lack of recognition as the case may be (Gibbs et al, 1986).

Clearly, VBF tissue resulted in hippocampal cholinergic reinnervation levels comparable to those in an intact brain. Considering that LBF tissue reinnervated so poorly it is interesting to consider why the combination of LBF and VBF tissue in group MBF then scored much more highly than would be expected by a simple averaging of the two tissue type effects. MBF tissue would be expected to produce a reinnervation score of ~60% by averaging the effects of its two constituent tissues, presuming both tissues survived approximately equally well after injection, yet group MBF scored a mean reinnervation level of 83% of normal. Perhaps most rats in group VBF achieved a maximal possible level of reinnervation with cell quantities in excess of that actually required despite the three small 1.5 µl aliquots used. Effectively diluting the concentration of VBF cells in group MBF by making a 50/50 mix of VBF and LBF tissues apparently yielded a mixture still capable of very high reinnervation levels. Alternatively, LBF cells, while causing poor cholinergic reinnervation alone, may supply neurotrophic support such that proportionally less VBF cells die in group MBF than in group VBF. In either case it is presumed that the cells responsible for the high reinnervation levels in group MBF are the VBF cells rather than the LBF cells.

The finding of the present study that septal tissue (group VBF) reinnervates to near normal levels, while NBM tissue (group LBF) yields poor reinnervation (at ~29% of normal levels) is consistent with the findings of Dunnett et al (1986) who found that septal tissue gave good transplant AChE-positive outgrowth while NBM tissue gave poor outgrowth (at ~20% of septal outgrowth levels). Nilsson et al (1988a) found results which are in conflict with those of Dunnett et al, and the present study, finding that NBM tissue gave good AChE-positive

reinnervation (to ~50% of normal levels) whereas septal tissue gave reinnervation equal to normal levels.

While the differential dissection schemes of Dunnett et al (1986), Nilsson et al (1988a) and the present study are obviously not identical, what is common to all three schemes is the attempted separation, within the cholinergically rich foetal basal forebrain, of the NBM cell population and the septal cell population. The use of the more restrictive areas VBF (septal tissue) and LBF (NBM tissue) in the present study was an attempt to more exclusively isolate the precursors of these cell populations and so be able to address this differential reinnervation discrepancy. As suggested earlier (see p 12 of introduction) a possible explanation for the discrepancy between Dunnett et al, and Nilsson et al was thought to be due to Dunnett et al taking a more rostral coronal section than Nilsson et al (see figure 1a), and so missing a caudal ACh precursor cell population, one which Nilsson et al could have captured by taking a more dorso-medial NBM area than their documentation would suggest and by using a coronal section which extended further in the caudal direction. Presumably like that of Nilsson et al, the present study used the coronal section shown in figure 1b and as such should have captured any more caudal ACh precursor cell populations. Yet the AChE positivity results of the present study support findings of Dunnett et al, and not Nilsson et al, in that they show poor reinnervation for lateral basal forebrain tissue (group LBF), and very good reinnervation for medial basal forebrain tissue (group VBF). This finding makes Nilsson et al's good reinnervation results for NBM tissue no less puzzling, since even with a more caudal coronal section (as in the present study) a reinnervation pattern like that of Dunnett et al was found.

The finding in the present study of preferentially reinnervated dentate gyrus areas particularly in the LBF tissue group was a result also reported in Nilsson et al (1988a) with NBM tissue, despite their finding of much better overall reinnervation. However, overall reinnervation in the LBF tissue group was generally much more like that reported in Dunnett et al (1986) who reported "patchiness" of reinnervation with darkly stained areas interspersed with blank areas.

Gibbs et al (1986) performed striatal tissue transplants into hippocampi deaffernated by transection of the fimbria-fornix and found reinnervation levels and patterns similar to those found with LBF tissue used in the present study. Striatal tissue is similar to LBF tissue in that both are laterally located in the coronal section, and both are known to contain NBM cells (Lewis and Cotman, 1983). Further, reinnervation patterns with Gibbs et al's striatal tissue showed preferentially darker staining of the dentate gyrus area as did LBF reinnervation.

All transplants masses in the present study were small and almost all exclusively contained within the hippocampus. In the rare cases that masses did extend into the ventricle they caused no displacement of the hippocampus or surrounding structures. This is in contrast with Nilsson et al (1988a) and Dunnett et al (1986) who both found sizeable disruptive transplant masses. The lack of such disruptive transplant masses in the present study is presumably due to the small aliquots (1.5 μl) used and the larger number of injection sites (3 bilaterally) spread over the dorsal hippocampus. Dunnett et al used only one bilateral transplant site and an aliquot of 3 μl, while Nilsson et al used 1.3 - 2.8 μl aliquots injected unilaterally at 2 sites.

T-maze testing.

Effects of fimbria-fornix lesions. After lesions to hippocampal circuitry (such as those caused by transection of the fimbria-fornix) rats often experience perseverative tendencies on spatial tasks (Dunnett et al, 1989; Jarrard, 1993). However, there was no evidence of any perseveration in any experimental group during T-maze testing in the present study.

The lesions produced a long term, severe deficit in spatial working memory (the T-maze trial-dependent arm discrimination), while lesions had no lasting effect on spatial reference memory (the T-maze trial-independent stem discrimination). Preoperatively, the reference memory component of the T-maze took longer for rats to learn than the working memory component, suggesting it was more difficult for the rats to solve, yet it was reference memory which recovered in all groups in the new testing room while working memory remained impaired at chance level throughout testing (see figures 5 and 6.1).

In the study by Dalrymple-Alford (1994), an identical T-maze testing procedure was used with rats with fimbria-fornix lesions, yet no improvement was found in reference memory performance, along with a persistent deficit in working memory. In that study rats were not trained preoperatively and encountered the T-maze for the first time after surgery. In their lesioned state the rats seemed incapable of learning to attend to extra-maze cues to solve the reference memory task. By contrast, the present work shows that if rats are tested preoperatively and then lesioned and moved to a new testing room with different extra-maze cues they can learn to attend to the new cues and show recovery of reference memory performance.

Rats must learn something during preoperative training which allows them to solve the reference memory task in the new testing room even after hippocampal lesions. But what ever it is they learn, they seem incapable of learning it after fimbria-fornix lesions. This is an interpretation which seems to make sense, but it is difficult to imagine exactly what it is the rats learn preoperatively. As the rats are forced to attend to extra-maze cues to solve the task, by virtue of the random physical swapping of the mazes, it is presumed that they orientate themselves in the room by attending to some prominent extra-maze feature, or features, and so are capable of discerning which maze they are on. The side of the stem which is open for either maze remains constant for any particular rat and so once the rat has worked out which maze it is on it has solved the reference memory task, as from this it knows which side of the stem will be open. Obviously, in the new testing room the prominent extra-maze feature to which they had previously attended will no longer be present, unless that feature was the other maze itself, since the positioning of the mazes relative to each other was invariant in both rooms. It seems unlikely that all rats would attend to the other maze as the extra-maze cue needed to solve the reference memory task. If there were a subpopulation which did not attend to the other maze, then these animals should not be able to solve the reference memory task in the new testing room, since all extra-maze cues are now different, and their post-lesion brains have a severe working memory deficit which should interfere with the learning of any new reference memory rule. For example, rats with a working memory deficit should at least have great difficulty learning the new rule of attending to the position of the door rather than the experimenter. However, after all groups began postoperative responding at approximately chance, presumably because of the delay since they had last been tested, and the trauma of surgery, all

lesion groups reacquired the reference memory task, and did so at the same rate as controls. This would suggest that what rats learn in preoperative training to solve the reference memory task is not a rule like "attend to extra-maze cue X", but something more abstract and subtle more akin to "find a feature in the room which is invariant with the side of the stem which is open". But given that what they learn in preoperative training is something like the abstract rule it is still necessary for them to actually attend to a new extra-maze cue. With fimbria-fornix lesions and without having learnt the abstract rule by way of preoperative training rats are incapable of learning to attend to any extra-maze cue (as demonstrated by Dalrymple-Alford's (1994) results), but they are capable of learning a new extra-maze cue with fimbria-fornix lesions if they learnt the abstract rule during preoperative training.

It is possible to resolve what rats are capable of learning and what they are not capable of learning before and after lesions in terms of different cognitive demands. Hodges et al (1990) claims that the extent of functional recovery in animals with lesions is crucially governed by the demands made on cognitive processes by different tasks, and states further that disruption of the cholinergic system is a disruption of attentional processes. Rats with fimbria-fornix lesions and disrupted cholinergic systems have greatly reduced attentional and cognitive resources. Therefore to learn the abstract rule with a fimbria-fornix lesion (ie, without preoperative training) is beyond the cognitive and attentional capacities of the rat. But to learn to attend to a new extra-maze cue with a lesion after having had preoperative training is more like trying to find something when you know what you are looking for, and so is within the reduced cognitive and attentional resources of the animal.

Complete disruption of working memory accompanied by sparing of reference memory after fimbria-fornix lesion ratifies predictions by Olton (1983), and supports his working/reference memory division which claims that the hippocampus is concerned with processing of working memory, while reference memories, such as how to solve the T-maze stem task, are stored elsewhere in the brain. Retrieval from reference memory is not affected by hippocampal/working memory disruption, but formation of new reference memories is affected since without working memory the animal lacks the cognitive and attentional resources to form new rules based on events in the world (Dalrymple-Alford, 1994).

Transplants. Fimbria-fornix lesion impairment was not ameliorated by any of the three cholinergic graft types. All transplant groups showed working memory performance at chance level and were not significantly different from each other or from lesion-only rats. Given the high level of qualitatively normal cholinergic reinnervation in at least the MBF group, the lack of behavioural recovery seems surprising. Particularly so when findings such as Hodges et al (1990) are considered which show full behavioural recovery of working memory in the presence of extensive septal graft deformation and destruction of the normal hippocampal laminar architecture. The hippocampal disruption present in Hodges et al was so extensive that even the authors stated that they found it hard to see how amelioration of the lesion effect could have taken place. The lesion site in Hodges et al was the basal forebrain, rather than the fimbria-fornix and the lesions were made with ibotenic acid, which is known to cause less severe behavioural deficits than fimbria-fornix transection (Jarrard, 1986). Dalrymple-Alford (1988, 1994) indicates that partial fimbria-fornix lesion following by cholinergic transplant can lead to greater

impairment than lesion alone. However, given the different behavioural effects of ibotenic acid lesions, partial fimbria-fornix lesions may not be comparable. As such the ibotenic acid lesions in Hodges et al could have had some degree of spared function after transplant. It is possible that lesion site and ibotenic acid versus fimbria-fornix transection are wholly responsible for the working memory recovery in Hodges et al in the presence of disruptive grafts, while no working memory recovery took place in the present study in the presence of qualitatively normal non-disruptive grafts. However, it seem likely that other confounding variables are also involved.

Reference memory showed a significant transplant effect: postoperatively, transplant group LBF scored significantly lower than controls ($p < 0.05$) in stem task testing. Of all groups, controls performed the best postoperatively at the reference memory task, attaining near perfect scores by the end of testing, and of all groups, LBFs performed the most poorly postoperatively at the reference memory task. All other transplant groups, and the lesion-only group, performed at intermediary levels. This relationship between group performances is best seen in the total scores graph, figure 6.2. Since all groups' performances increased at nearly the same rate over sessions there was no interaction effect (see figure 6.1).

LBF group scores were significantly different from controls, suggesting that it is the combination of a lesion followed by an LBF tissue transplant that has a deleterious effect on reference memory performance. While LBF transplants gave the poorest cholinergic reinnervation the LBF group's poorer performance at the reference memory task cannot be interpreted as one of cholinergic status of graft per se, as the LBF group did

not score significantly differently from the lesion-only group. For the poorer postoperative reference memory performance to be directly attributable to graft effect LBF group scores would need to be significantly lower than lesion-only scores to demonstrate that an LBF transplant exacerbated the effect of a lesion; this was not the case.

DRL testing.

All lesion groups showed impairment at the DRL-20 task scoring only ~5% correct compared to normal controls which scored ~30% correct (see figure 7). No transplant amelioration of lesion effect was evident in any group.

Dunnett et al (1989) however, did find recovery of DRL-20 responding after fimbria-fornix lesion and septal grafts. While Dunnett et al used 13 mm CRL donor tissue and a lesion-transplant delay of 10 days, comparable to the 12/13 mm CRL tissue and the 11 to 15 day delay used in the present study, his methods did differ in at least two important respects.

First, the tissue area Dunnett et al used for transplantation was the "standard" septal area as shown in figure 1b, while the tissue areas used in the present study comprised a differential dissection of a different forebrain area into the areas ventro-medial basal forebrain (area VBF), and lateral basal forebrain (area LBF) shown in figure 1f. Tissue areas VBF and LBF were an attempt to attain transplant material which contained as exclusively as possible the NBM cell population and the ventral septal area within the cholinergically rich basal forebrain. The dorsal septal area was not included in the VBF tissue as it is the ventral area which is

known to be the most cholinergically rich. Despite the restrictive area taken as VBF tissue extremely good reinnervation resulted nonetheless. Tissue area VBF in the present study and Dunnett et al's septal area are both similar and comparable in their excellent cholinergic reinnervation capacities. Even the mixed group MBF which contained half as many VBF cells, at least at the time of injection, still gave very good cholinergic reinnervation to a mean of 83% of normal levels. This would suggest that Dunnett et al's behavioural recovery at the DRL-20 and the present study's lack of a behavioural recovery is not dependent on the cholinergic reinnervation capacity of the transplant tissues used in either study, and is more likely to be due to the presence of other cell types in Dunnett et al's less exclusive septal area. Dunnett et al's "standard" septal area contained a dorso-medial area and ventro-lateral areas which are not included in tissue area VBF, and so these areas could have imported other cell populations which contributed to the behavioural recovery reported by Dunnett et al.

Second, Dunnett et al performed an aspirative lesion of only the fimbria-fornix, sparing the cingulate bundle and sagittal sinus, while the lesion in the present study was of the fimbria-fornix and overlying cingulate cortex, destroying the cingulate pathway. However, this does not explain why Dunnett et al found behavioural recovery at the DRL task while the present study did not, since both achieved high levels of cholinergic reinnervation, considered the single most important causal factor in transplant induced behavioural recovery. There is evidence to suggest that the fimbria-fornix and cingulate cortex fibre pathways carry different kinds of inputs to the hippocampus. The fimbria-fornix has an important modulatory control function of such things as motivation, arousal, and autonomic states (Apostal and Creutzfeld, 1974), while the cingulate

pathway may have a more specifically informational role (Swanson, 1979). Hippocampal suspension injection transplants restore cholinergic reinnervation, and normal, or near normal laminar patterns within the hippocampus. This normalisation is also thought to extend to cholinergic modulation of the hippocampus, and perhaps in the presence of intact cingulate afferents this allows the transplant rats to perform the DRL task with an efficacy comparable to controls. However, without intact cingulate afferents, as in the present study, modulation may be restored but without specific informational inputs the rats may not be capable of timing their bar pressing responding normally and so are unable to perform the DRL task. However, Nilsson et al (1987) used a lesion similar to that in the present study and found recovery of spatial navigation with septal transplants, suggesting that on balance the neurochemical nature of the transplanted tissue is likely to be more important than any particular lesioned pathway.

Since modal Inter-Response Times (IRTs) across sessions for the DRL-20 indicated that all lesion rats were responding at ~13 s, far short of the 20 s required to perform the DRL-20 to any level of proficiency, the waiting period was reduced to 10 s (see figure 7 for DRL-10 results). Individual session block contrasts of DRL-10 testing confirmed lesion-only rats to be improving over sessions, such that by session block 7 their performance was no longer significantly different from controls - being intermediary at ~45% correct between that of controls at ~60% correct and that of transplant groups at ~32% correct. Transplant groups also showed improving performance over sessions at the DRL-10 task, however, transplant group scores were always significantly different from controls.

The ability for lesion-only rats in the present study to manage some level of proficiency at DRL-10, but not DRL-20 is consistent with other work

showing rats with hippocampal damage as able to perform adequately on low DRL schedules but not ones requiring greater delays since lesion rats have difficulty withholding responding for more than 10 to 15 s (Ellen and Aitken, 1970). The modal DRL-20 IRTs of ~13 s found for all lesion groups in the present study lies within this 10 to 15 s maximum response withholding range for hippocampally damaged rats, and confirms that transplants have had no effect in restoring normal DRL-20 responding behaviour.

In addition to the lack of evidence for any ameliorative effect of transplants on DRL-20 performance there is evidence for slight deleterious effect in the fact that individual session block contrasts on the DRL-10 task show that lesion-only scores increase to the point where they are no longer significantly different from controls, while all transplant groups always remain significantly different from controls. It seems reasonable that a deleterious effect of transplantation may show up in DRL-10 but not DRL-20 results since correct responding was so low on the DRL-20 task as to constitute a floor effect, whereas DRL-10 appears to be a more sensitive measure of differences in experimental groups, sensitive enough to pick up a slight deleterious transplant effect.

Activity testing.

All lesion groups showed increased spontaneous activity compared to controls, showing no evidence of graft induced amelioration of hyperactivity in any group. This failure of amelioration of hyperactivity in the present study using 12/13 mm CRL transplant tissue is consistent with findings by Dunnett et al (1989), and Cassel et al (1991). Dunnett et al found that while younger transplant tissue (13 mm CRL) gave the best

behavioural recovery on a DRL task after fimbria-fornix lesion it was older tissue (17 and 22 mm CRL) which best ameliorated spontaneous hyperactivity. Similarly Cassel et al found after fimbria-fornix lesion that is was older tissue (16 mm CRL, E 16) rather than younger tissue (12 mm CRL, E 14) that improved open field habituation as measured by a significant within trial decline in ambulatory activity.

Dalrymple-Alford (1994) found normalisation of activity in the Hughes (1968) exploration box after fimbria-fornix lesion and standard septal area transplantation with tissue of an average size of 16.5 mm CRL. While Dalrymple-Alford's transplant tissue included young donor material of the size 13 and 14 mm CRL, all hosts received a mixture of a number of different ages of tissue and included many hosts which received considerably older tissue of the ages 18 and 22 mm CRL. As such any tissue age effect on amelioration of activity is confounded by the mix of tissue ages, but inclusion of older material and the finding of amelioration of activity is consistent with previously discussed findings (Cassel et al, 1991; Dunnett et al, 1989) and those of the present study.

Group LBF showed evidence of increased behavioural impairment on the reference memory task, and all transplant groups showed evidence of increased behavioural impairment at the DRL-10 task. Despite this however, group LBF did not show any significantly higher level of hyperactivity on either activity measure (crossings or total beam breaks). Even habituation over bins during the activity testing session showed no differential effect between transplant groups.

Factors involved in graft success.

Tissue age. Why should older tissue generally ameliorate hyperactivity and not cognitive or memorial functional deficits, while younger tissue tends to ameliorate cognitive functional deficits and not hyperactivity? A possible explanation could be in terms of differential survival of cell types in young and old tissue. Perhaps the subpopulations of cells responsible for different ameliorative effects have different window periods where transplantation would be optimally effective. As different parts of the brain develop and functionally mature at different times, and different neural systems are responsible for different aspects of behaviour, this idea seems sensible. Young tissue of size 11-13 mm CRL may correspond to the window period where the subpopulation of cells responsible for the amelioration of functional deficits are at their most robust, and so will be more likely to differentially survive the rigours of transplantation. Where as older tissue of the size ~17-22 mm CRL may be nearer the optimal window period for the subpopulation of cells responsible for the amelioration of hyperactivity, and so these cells will differentially survive. Such an idea suggests the possibility of mixing young and old tissue together for purposes of transplantation to gain amelioration of both functional deficits and hyperactivity. Other factors affecting behavioural recovery such as lack of preoperative training (Dalrymple-Alford, 1994), and the nature of the lesion would naturally have to be controlled for.

The involvement of other neurochemical systems. The lack of behavioural improvement on any measure in light of an optimal lesion-transplant delay, optimally aged tissue (Dunnett et al, 1989), and excellent

cholinergic reinnervation in the present study is strong evidence for the view that graft induced cholinergic reinnervation is a necessary, but not by itself a sufficient factor for restoration of normal behavioural functioning (Cassel et al, 1992). Other neurochemical candidates for involvement exist. Cassel et al (1991) found results which suggest that behavioural effects of transplants after fimbria-fornix lesion could be due to differential survival of GABAergic neurones in transplanted septal-diagonal band tissue. Springer and Isaacson (1982) found evidence for the role of dopamine in normal hippocampal function, demonstrating changes in the level of dopamine and its utilisation after hippocampal damage. Jarrard (1986) found evidence for noradrenergic inputs to the hippocampus. But perhaps the most convincing evidence for other neurochemical involvement in memory function is presented in a series of experiments by the Nilsson et al (1990) investigating the role of serotonin.

Nilsson et al (1988b) produced cholinergic and serotonergic denervation of the forebrain by intraventricular injection of 5,7-dihydroxytryptamine (5,7-DHT) and radiofrequency lesion of the septum. They found that while serotonergic depletion alone did not produce any significant deficits in the Morris (1981) water maze task, combined serotonergic and cholinergic denervation produced deficits which were even more severe than cholinergic denervation alone. That is, serotonergic depletion appeared to greatly potentiate behavioural deficits when in combination with cholinergic depletion, an effect which was persistent over time. In a further study Nilsson et al (1990) attempted to ameliorate the behavioural deficits of combined cholinergic-serotonergic denervation of the forebrain with suspension transplants of either serotonergically rich raphe tissue, septal tissue or a combination of both tissues. Only the combination

transplant of raphe and septal tissue showed any behavioural improvement, while all other lesion groups showed performance which was significantly impaired compared to controls. Histological analysis of transplant groups in this study showed that rats with only a septal graft showed normal AChE-positivity, but were devoid of serotonin-positive fibres. In rats that received a combination graft of septal and raphe tissue complete AChE-positive and serotonin-positive reinnervation was found. This series of experiments by Nilsson et al offers convincing evidence for serotonergic interaction with the cholinergic system in memory function.

While Nilsson et al (1988b, 1990) used 5,7-DHT and radiofrequency lesion of the septum to achieve neurochemical denervation rather than fimbria-fornix lesion such an idea of serotonergic interaction is also consistent with many previous behavioural findings after fimbria-fornix lesion (including Nilsson et al (1987), Dalrymple-Alford (1994) and the present study). In Nilsson et al (1987) some rats did not show behavioural recovery after fimbria-fornix lesion and septal transplantation despite extensive AChE-positive reinnervation, while others showed partial recovery. Interpreting this result now in the light of serotonergic involvement seems to make sense. Perhaps the rats which did experience behavioural recovery after septal transplantation received a lesion which left the hippocampal serotonergic system largely intact and so these rats required only cholinergic reinnervation to attain recovery, while the rats which experienced no recovery after septal transplantation received a lesion which caused more complete serotonergic denervation, and so cholinergic reinnervation alone was not sufficient to cause behavioural recovery (Nilsson et al, 1987).

Likewise, the present study showed excellent AChE-positive reinnervation in group VBF and good reinnervation in group MBF, yet no behavioural recovery was evident. Perhaps all fimbria-fornix lesions in the present study also caused extensive serotonergic denervation, and so septal transplantation alone was not enough to restore function. Nilsson et al (1990) has demonstrated the possibility of recording normal AChE-positivity in the presence of complete serotonergic depletion, but further serotonergic histology would be needed to confirm such a state of affairs in the present study. However, such an explanation does not explain the deleterious effects of transplants in the present study, namely the LBF group's performance at the reference memory task and all transplant groups' performance at the DRL-10 task. A lack of serotonin may prevent behavioural recovery with septal transplants alone, but it would not be expected to exacerbate functional deficits which already exist.

In addition, other findings are also problematic for the idea of a simple cholinergic/serotonergic interaction in memory function. Murtha and Pappas (1994) found, as did Nilsson et al (1988b), that serotonergic denervation of the hippocampus had no effect by itself on Morris (1981) water maze performance. However, contrary to findings by Nilsson et al, Murtha and Pappas found combined damage of hippocampal cholinergic and serotonergic afferents did not severely affect spatial memory, thus failing to support the idea that serotonergic depletion potentiates behavioural deficits caused by cholinergic denervation.

If serotonergic depletion is responsible for the lack of behavioural recovery in the present study it would be expected that combined septal/raphe transplants would succeed in ameliorating such deficits. However, a study conducted by Cassel et al (1992) suggests otherwise. Cassel et al performed aspirative lesions of the fimbria, dorsal fornix and

parts of the overlying structures, a lesion very similar to that used in the present study, followed by septal transplants alone, raphe transplants alone, and combination septal/raphe transplants. No transplant type produced significant behavioural recovery despite restoration of cholinergic and serotonergic markers.

The involvement of any neurochemical system or systems with memory function would involve theoretical and experimental considerations including much of that which has just been considered for serotonin. At present no single non-cholinergic neurochemical is unproblematically implicated.

Concluding remarks. The poor to excellent cholinergic reinnervation in the present study showed no concomitant differential behavioural recovery, whether lesion animals were devastatingly impaired, as on the working memory task, or showed near normal performance, as on the reference memory task.

Considering the variables controlled for in this study, it is strong evidence for the involvement of other neurochemical systems in memory function. Such involvement of non-cholinergic neurochemicals seems more likely to be via a "cocktail" of interacting neurochemicals rather than via a single neurochemical such as serotonin.

The deleterious effect on behaviour of lesions and transplants in this study is further evidence that the effects of cholinergic rich grafts are not wholly ameliorative, neither are they sufficient for amelioration.

Appendix.

Table 1. F, p and df values for DRL-10 individual block contrasts.

df = 1,35		5	6	7	8	9	10
VBF/Control	F	16.94	10.92	8.90	7.32	6.44	10.28
	p	0.001	0.005	0.01	0.02	0.02	0.005
LBF/Control	F	18.40	13.84	11.90	9.20	6.08	10.45
	p	0.001	0.001	0.002	0.005	0.02	0.005
MBF/Control	F	15.57	9.41	7.52	6.62	5.35	7.32
	p	0.001	0.005	0.01	0.02	0.03	0.02
VBF/L Only	F	< 1	< 1	1.43	< 1	1.68	2.04
	p			0.10		0.10	0.10
LBF/L Only	F	< 1	< 1	2.98	1.10	1.57	2.25
	p			0.10	0.10	0.10	0.10
MBF/ L Only	F	< 1	< 1	< 1	< 1	1.20	< 1
	p					0.10	
L Only/Contrl	F	12.89	9.67	3.17	4.09	1.57	3.18
	p	0.001	0.004	0.09	0.051	0.22	0.09

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